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Development of a quantitative high-performance liquid chromatography–photodiode array detection measurement system for phenolic acids

Rebecca J. Robbins [a,*], Scott R. Bean [b]

[a] *Food Composition Laboratory, Beltsville Human Nutrition Research Center, Agricultural Research Center, US Department of Agriculture, Building 161, BARC, East Beltsville, MD 20705-3000, USA*
[b] *Grain Marketing and Production Research Center, Agricultural Research Center, US Department of Agriculture, 1515 College Avenue, Manhattan, KS 66502, USA*

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Abstract

A quantitative high-performance liquid chromatography–photodiode array detection method separating 16 phenolic acids was achieved. Six columns and several mobile phases were investigated. Resolution was achieved with a high-purity silica Phenomenex Luna C_{18} column (150 mm × 4.6 mm, 5 μm) and a binary gradient consisting of CH_3OH–water (with 0.1% formic acid) and flow rate set at 0.7 ml/min. Acids were detected and quantitation performed at wavelength representing the lowest energy λ_{max} for individual acids. Extraction procedure from wine was optimized and yields ranged from 79 to 87% based on internal standard recovery. To confirm our quantitative results, identical samples were analyzed both in-house and by a collaborating laboratory. Correlation of two data sets generated linear regression equations that approached unity (0.93–0.98) and R^2 values ranging from 0.990 to 0.999.
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1. Introduction

Epidemiological evidence suggests that the consumption of fruits and vegetables may reduce the risk of some forms of cancer, cardiovascular disease and occurrence of strokes [5,9,11]. Current thought links the high-antioxidant content of plant-based foods with the inhibition of these oxidative damage diseases. “Phenolics” are one class of plant metabolites that demonstrate antioxidant activity. The name ‘phenolics’ encompasses a large variety of naturally occurring molecules currently classified according to number of phenol subunits (Fig. 1). A monomeric subclass consists of the phenolic acids. Although these acids demonstrate comparable antioxidant activity to their polymeric counterparts (i.e. flavonoids), less is known concerning their health or antioxidant benefits [12,20].

Accurate analysis of foods and generation of reliable food composition databases are needed to establish associations between dietary intake and disease prevention by phenolic acids. Appropriate analytical methods are critical to the development of such food databases. In the literature, the impetus for developing analytical methods for phenolic acids has been multifaceted and originates from the interest in their biological roles [2,21], organoleptic properties and food quality [7,13,19] and are mostly of a qualitative nature. Few quantitative methods have been established or devised specifically for the purpose of compiling nutritional or food databases by generating accurate food composition data [16].

To be able to analyze commonly consumed foods in a routine fashion requires robust analytical methods that generate quality data (i.e. accurate and precise quantitative measurements). Although not all phenolic acids exist in all foods [18], development of a single high-performance liquid chromatography (HPLC) method which could be employed to simultaneously identify and measure

* Corresponding author.
E-mail addresses: robbins@bhnrc.usda.gov (R.J. Robbins), scott@gmprc.ksu.edu (S.R. Bean).

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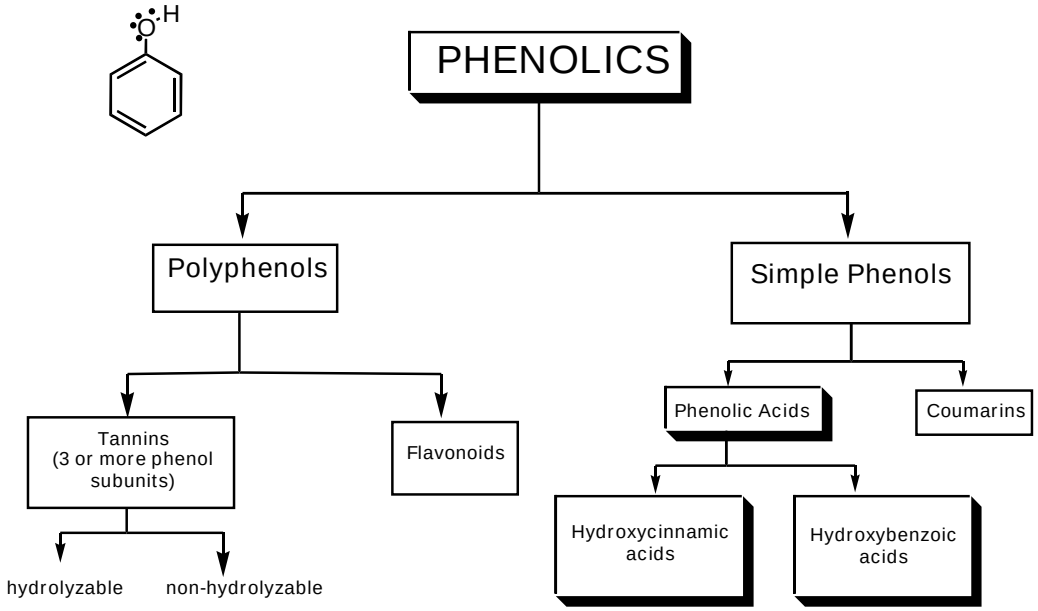


Fig. 1. Classification scheme of phenolics according to number of phenol subunits.

prominent phenolic acids in a variety of foods would be desirable. Furthermore, designing a chromatographic system which can be interfaced with various detection systems would broaden the applicability of the measurement system.

In this report, we describe an HPLC system for the separation of sixteen prominent phenolic acids with photodiode array detection (DAD) where the chosen mobile phase is compatible with MS detection. Since no standard reference materials are available, cooperation with another laboratory (i.e. exchanging samples) provided a means to compare and assess analytical results. Finally, the developed method was used to analyze several different wine samples to demonstrate its utility.

2. Experimental

2.1. Reagents

Acetonitrile, formic acid, ethyl acetate, methanol, acetic acid, HCl were all of analytical or HPLC grade and purchased from Fisher (Fairlawn, NJ, USA). Magnesium sulfate and trifluoroacetic acid were purchased from Aldrich (Milwaukee, WI, USA). All phenolic acids standards (gallic, vanillic, ferulic, gentisic, *ortho*-, *para*-, *meta*-coumaric, caffeic, protocatechuic, syringic, sinapic, chlorogenic, *p*-hydroxybenzoic, syringealdehyde and protocatechualdehyde) were obtained from Sigma (St. Louis, MO, USA) except for 2,3,4-trihydroxybenzoic acid which was purchased from Fluka (Buch, Switzerland). Deionized water (18 Ω) was prepared using a Millipore-Milli-Q purification system (Millipore, New Bedford, MA, USA).

2.2. Sample preparation

2.2.1. Food samples

Wines were purchased from local stores: (1) Sutter Home Zinfandel 1999 Napa, CA, USA; (2) Vendange Merlot 2000 Woodbridge, CA, USA; (3) Gallo Merlot 2001 Modesto, CA, USA; (4) Chateau Montet Bordeaux 2001 Gironde, France.

2.2.2. Extraction

Extraction conditions were a modification of the method reported by Rodriguez et al. [15]. Ethyl acetate (5 ml) was added directly to the wine (5 ml). No pH adjustments were made. Mixture was sonicated (FS-20, Fisher Scientific, Pittsburgh, PA, USA) for 20 min. Layers were separated and organic layers were combined, filtered through a 0.22 μm syringe filter (Millipore, Bedford, MA, USA). Organic solvent was dried with $MgSO_4$ (150 mg), evaporated under a stream of nitrogen and redissolved in 1 ml of methanol–deionized water containing 0.1% (v/v) formic acid (50:50). Sample was filtered once again prior to injection into HPLC.

2.3. Instrumentation and chromatographic conditions

The separation of phenolic acids was performed with a Beckman Coulter System Gold (SG) HPLC system equipped with a solvent-pump module (SG 126), a photodiode array detector (SG 168), autosampler (SG 508), and Alltech 530 column heater. Data analysis was carried out using 32 Karat Software through Windows NT.

Six columns were initially tested: Alltech Absorbosphere C_{18} (150 mm × 4.6 mm, 3 μm, Deerfield, IL, USA), Phenomenex Luna C_{18}-high-purity silica (150 mm × 4.6 mm,

Table 1
Retention times, peak identification, UV absorbance maxima, (λ_{max}) and λ analysis for the phenolic acids

No.	Acid	Retention time (min)	UV λ_{max} (nm)	$\lambda_{anal.}$ (nm)
1	Gallic	10.5	217, 272	270
2	Protocatechuic	19.1	218, 261, 295	295
3	2,3,4-Trihydroxy benzoic	20.8	227, 267	267
4	Protocatechuic aldehyde	22.7	232, 280, 311	310
5	*p*-Hydroxybenzoic	28.7	202, 257	260
6	Gentisic	33.3	213, 239 (s), 332 (m), → 370	325
7	Vanillic	37.0	219, 261, 294, → 320	260
8	Chlorogenic	37.8	243, 325	325
9	Caffeic	39.4	220, 240 (br), 294 (ps), 325	325
10	Vanillin	41.6	232, 280, 310	310
11	Syringic	42.5	220, 275, → 328	270
12	Syringealdehyde	46.2	225, 308	310
13	*p*-Coumaric	52.1	229, 312, → 361	310
14	Ferulic	56.9	218, 236 (br), 294 (ps), 324	325
15	Sinapic	59.7	238, 326	325
16	*m*-Coumaric	62.5	216, 233, 278	278

(→) Extends out to; (s) shoulder; (m) moderate absorbance; (br) broad; (ps) pre-shoulder. Separation is shown in Fig. 3.

5 μm, Torrance, CA, USA), Phenomenex Luna Phenyl-Hexyl (150 mm × 4.6 mm, 5 μm, Torrance, CA, USA), Phenomenex Luna C_8 (150 mm × 4.6 mm, 5 μm), Phenomenex Aqua C_{18} (150 mm × 4.6 mm, 5 μm), Rainin Microsorb-MV C_{18} (250 mm × 4.6 mm, 5 μm, Woburn, MA, USA). A Phenomenex C_{18} ODS, Octadecyl guard column was used (4 mm × 3.0 mm) with all columns.

Resolution of the 16 acids and subsequent analytical investigations were carried out with the Phenomenex Luna C_{18} high-purity silica (150 mm × 4.6 mm, 5 μm) using methanol (B) and 0.1% aqueous formic acid (A) as the binary solvent system. The flow rate was set at 0.7 ml/min. Column and guard column were thermostatically controlled at 25 ± 0.3 °C. Injection volume was set to 30 μl. Autosampler tray was set to 15 °C. Wavelengths used for detection and quantification of the individual phenolic acids are listed in Table 1. Concentrations in trace present in Fig. 3 were (reported in mg/l): **1** (2.38), **2** (4.42), **3** (6.43), **4** (4.76), **6** (9.52), **7** (5.08), **8** (15.71), **9** (4.92), **10** (20.0), **11** (5.19), **12** (4.76), **13** (4.69), **14** (3.94), **15** (4.76), **16** (4.76).

Collaborative work was performed on an Agilent 1100 HPLC system with DAD. The column was maintained at 25 °C with the Agilent thermostating system compartment. Column was a Phenomenex Luna C_{18}. Sample preparation is the same as described in Section 2.2.2. Wine samples (100 ml) were spiked with a known amount of a given phenolic acid. For the Vendange wine sample, 9.6 ml of a caffeic acid stock solution of 104.2 mg/l was added to a volumetric flask. The volume was then brought to 100 ml with the wine. The sample was then divided into two equal amounts (50 ml). The Bordeaux and Zinfandel were treated in the same fashion except that ferulic (9.5 ml of 52.46 mg/l) and vanillic (13.0 ml of a 53.37 mg/l) were the standards added, respectively. A set of three duplicate wine samples were analyzed in house and a second set of three wine samples were analyzed in a collaborator's laboratory.

2.4. Quantitation

2.4.1. Calibration curves

The external standard method was the technique used for quantitation. Peak areas from HPLC chromatogram were plotted against the known concentrations of stock solutions of varying concentrations. Slope, intercept and other statistics of calibration lines were calculated with linear regression programs using Microsoft Excel version 9.0. Equations generated via linear regression were used to establish concentrations for foods and standards solutions. A wide concentration range of the standards was assessed since varying amounts are present in different foods (~0–100 mg/l).

2.4.2. Preparation of standards solutions

For each acid, two initial stock solutions were made by accurately weighing out the commercially standard with a Mettler Toledo AX205 Delta Range balance followed by dissolution in 1 l of 10% (v/v) methanol in deionized water. Solutions used for calibration were prepared by dilution of the two initial stock solutions. Dilution of one gave a series of solutions with concentrations of near 90.0, 70.0, 50.0, 30.0, 10.0 mg/l. Dilution of the other stock solution gave concentrations of 100.0, 80.0, 60.0, 40.0, 20.0 mg/l. A Rainin pipette (5 ml) used for volumetric measurements was calibrated prior to use (volume of deionized water was weighed on an Ohaus Analytical Plus balance). Mean areas ($n = 4$) generated by five to seven of the standard solutions were plotted against concentration to establish calibration equations. The remaining diluted stock samples were used to assess the accuracy of the calibration equation. Large volume stock solutions were stored at −60 °C. Working range calibration standards were stored at 4 °C until needed or at normal refrigerator temperature for overnight storage. A mixture of standards was used for the HPLC method development. Several stability assessment experiments were run

on the standard solutions of phenolic acids. Reproducibility of area and retention times were examined. Six replicates (approximately 8 h HPLC time) were run at room temperature and with the auto-sampler tray set to 15 °C. Stability was also tested over a period of 3 days at room temperature and at 4 °C.

2.4.3. Limits of detection and quantitation

Limits of detection (LODs) were determined by running 30 blanks, multiplying the standard deviation of the peak area by three and converting area to concentration of phenolic acid. For limits of quantitation (LOQs) the standard deviation was multiplied by fifteen and then converted to concentration [6].

3. Results and discussion

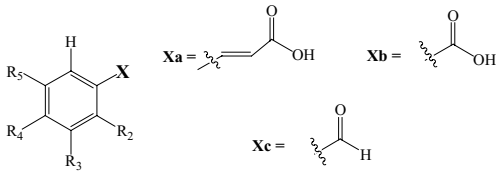
3.1. Method development

Phenolic acids contain two distinguishing constitutive carbon frameworks: the hydroxycinnamic (**Xa**) and hydroxybenzoic (**Xb**) structures (Fig. 2). Although not technically phenolic acids, several aldehyde analogues (**Xc**) are also referred to as phenolic acids (e.g. **4**, **10**, **12**). The structural differences between various phenolic acids are small, originating from the number and positions of the hydroxyl and methoxy groups on the aromatic ring.

Our initial chromatographic separation experiments were performed on six different columns with a standard mixture containing 7 phenolic acids (**1**, **2**, **7**, **9**, **11**, **13**, **14**). Although reversed-phase columns (C_{18}) are commonly used in chromatographic systems, few investigators explain their choice of column [3,22]. To remain consistent with developing a robust method, standard C_8 and C_{18} columns of varying particle size (3 and 5 μm) and column length (150 and 250 mm) were chosen, however, we tested several alternate stationary phases as well. The Phenomenex Luna Phenyl-Hexyl was investigated to potentially exploit π–π interaction between the aromatic acids and stationary phase to enhance resolution. Since the starting mobile phase was 95% aqueous, the Phenomenex Aqua was also examined. Initial chromatographic runs were gradients that ranged from 5% organic phase to either 30 or 100% over 50 min. Six binary solvent systems were investigated consisting of an organic phase (either acetonitrile or methanol) and an aqueous phase containing 0.1% of either trifluoroacetic, acetic or formic acid.

In these early traces, poor resolution of the seven acid stock solutions was observed with the Aqua, Phenyl-hexyl and Alltech columns. Better resolution was obtained with methanol in the mobile phase for the remaining columns (Phenomenex Luna C_{18}, Phenomenex Luna C_8, Rainin Microsorb-MV C_{18}). With all columns except the Phenomenex Luna column, resolution of acids **7**, **9** and **11** was problematic.

Typical acid additives in the mobile phase are sulfuric, phosphoric, formic, acetic and trifluoroacetic acids [14]. Although acetic acid is most often chosen as the acid additive, our early scout runs demonstrated poor peak shape with this acid. This is not surprising since the pK_a of acetic acid is in the same range as that of the phenolic acids (**4**–**5**) and will



R_2	R_3	R_4	R_5	X	Common Name
H	-OH	-OH	H	a	Caffeic Acid
H	H	-OH	H	a	*p*-Coumaric Acid
H	$-OCH_3$	-OH	H	a	Ferulic Acid
H	$-OCH_3$	-OH	$-OCH_3$	a	Sinapic Acid
H	-OH	H	H	a	*m*-Coumaric Acid
H	-OH	-OH	-OH	b	Gallic Acid
H	-OH	-OH	H	b	Protocatechuic Acid
-OH	-OH	-OH	H	b	2,3,4-trihydroxybenzoic Acid [a]
H	H	-OH	H	b	*p*-Hydroxybenzoic Acid
-OH	H	H	-OH	b	Gentisic Acid
H	$-OCH_3$	-OH	H	b	Vanillic Acid
H	$-OCH_3$	-OH	$-OCH_3$	b	Syringic Acid
H	-OH	-OH	H	c	Protochatechuic aldehyde
H	$-OCH_3$	-OH	H	c	Vanillin
H	$-OCH_3$	-OH	$-OCH_3$	c	Syringealdehyde

[a] 2,3,4 trihydroxybenzoic acid is not a naturally occuring phenolic acid. It is used as an internal standard

Fig. 2. Structures and substitution patterns for prominent phenolic acids.

not dramatically offset the equilibrium from carboxylate to carboxylic acid. This acid can, however, protonate the phenolic hydroxyl groups making it an effective additive for polyphenols (the pK_a of the phenolic proton of flavonoids hovers around 10).

Trifluoroacetic acid and formic acid gave similar results with respect to peak shape. Formic acid was eventually chosen to enhance the flexibility of the detection method—potential transferability to MS detection (MS detection methodology is currently being developed in our laboratory). The most common flow rate for a 4.6 mm i.d. column is 1 ml/min. A lower flow rate (0.7 ml/min) was chosen to enhance resolution as well as increase flexibility to alternate detection systems. Column temperature was set at 25 °C to emulate typical room temperature yet ensure constant temperature stability. Although the retention times decreased with increasing temperature (25–45 °C, increments of 5 °C), several analytes shifted elution order and co-eluted (e.g. **7** and **9**; **14** and **15**; **8** and **9**) at higher temperatures.

An additional 9 phenolic acids were added to the stock solution (total 16). With the initial mobile phase concentration set at 5% B followed by a slow increase to 30% B over 50 min and an isocratic step (30% B) for 15 min, separation of the 16 aromatic acids was achieved with good resolution. A chromatogram showing the separation of the 16 acids is shown in Fig. 3.

At 65 min the mobile phase concentration was dropped back to 5% B and held for 10 min for column equilibration. Standard solutions were repeatedly injected ($n = 7$) to confirm reproducibility of peak area and retention times. R.S.D.s ranged from 0.4 to 1.2% for areas and 0.26 to 0.50% for retention times. Peak identification, retention times obtained (at 25 °C), wavelength used for analysis and λ_{max} for phenolic acids are given in Table 1.

In the literature, the single most common wavelength used for detection of phenolic acids is 254 nm [14]. This is not the overall λ_{max} and perhaps was chosen due to convention based on the strong energy line from earlier lamps. Food matrices contain other UV active organic molecules (many absorbing ~200–250 nm). Therefore, to enhance selectivity without sacrificing sensitivity, the longest wavelength λ_{max} was chosen for detection of the analytes (e.g. monitored for **2** at 295 nm instead of 260 nm).

Data were also collected at 270 nm since it gave the best signal to noise response for all acids simultaneously as well as assisted in distinguishing between hydroxycinnamic and benzoic acids. Phenolic acids with the benzoic acid carbon framework (**Xb**) have their λ_{max} in the 200–290 nm range (Table 1). One exception is for **6**, which has a strong absorbance that extends to 355 nm. The cinnamate derivatives (**Xa**), due to the additional conjugation, show an additional broad absorbance band from 270 to 360 nm.

The relatively small structural features of the phenolic acids make a dramatic difference in retention on the C_{18} stationary phase as illustrated by **9** (t_R 39.4 min) and **14** (t_R 56.9 min) where the only difference in the molecule is a single methyl group. Regio-isomers also exhibit different retention behavior (e.g. **2** versus **5**). In our method, the smallest peak to peak separation is 0.9 min between **10** and **11**. They can easily be quantified at different wavelengths (310 versus 270) since 10 absorbs out to 350 nm where as 11 is no longer detectable above 290 nm.

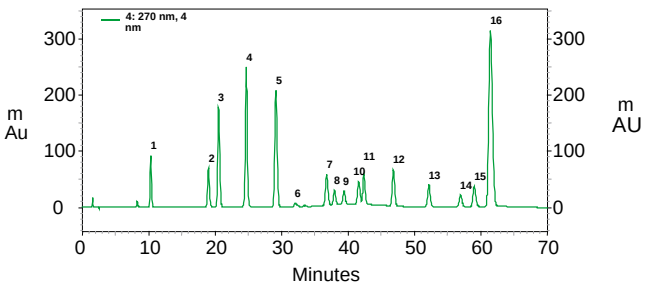


Fig. 3. Chromatogram of a solution of 16 phenolic acids monitored at 270 nm. Chromatographic conditions are described in the text. See Table 1 for peak identification.

3.2. Stability of standard solutions

No standard reference material for phenolic acids is commercially available at this time; therefore, a mixture of phenolic acids is being developed as a laboratory standard. The short and long-term stability of the phenolic acids in the methanolic solution were assessed. Except for caffeic (**9**), the R.S.D.s (area) for all acids tested, under various conditions were small. Over a period of approximately 8 h at room temperature the R.S.D.s ranged from 0.7 to 1.0%. At 15 °C over the same period of time the variation was 0.4–0.7%. For a period of 3 days at room temperature, the concentrations deviations ranged from 0.16 to 2.6% and over a period of 3 days stored at 4 °C, the variations were 0.12–1.6%. Acid **9**, however, started to decompose within 1 day at room temperature (R.S.D. = 9.1% within 8 h). Stability experiments for **9** were repeated under various conditions (e.g. shielded from light, the presence of oxygen and the temperature was controlled). Lowering the temperature made a significant impact. Stored at 15 °C (over the 8 h run time) caffeic acid (**9**) showed a R.S.D. of 0.66%. After 3 months when stored in the refrigerator, again only the stock solution of **9** showed signs of decomposition. Systematic precautions (amber vials, fresh standards and temperature control) were taken with all standard solutions and food samples to ensure stability. When stored at −60 °C, no decomposition was observed after 6 months.

3.3. Calibration

A major goal of a quantitative measurement system—for food composition—is the accurate collecting and reporting

of concentration values. Calibration curves were established and uncertainties assessed for prominent phenolic acids at concentration ranges that are expected in foods [4]. Linearity was observed over a wide range for all acids (0–100 mg/l for all acids). Both unweighted linear regression (LR) and log–log (LL) plot equations were generated. Correlation coefficients for the curve fittings ranged from 0.9985 to 0.9999 and 0.9991 to 1, respectively.

To assess accuracy, 5 of the 10 standard solutions (of known concentrations) generated, were used to establish the calibration curve and the other five standards were run as unknowns. Since correlation coefficients inherently reflect a better fit at higher concentrations versus at low [8] concentrations, we chose to further investigate accuracy at the lower end of our concentration curves and ran a low concentration (1 mg/l) sample as well.

A comparison of errors obtained using LR versus LL plots generated equations for the phenolic acids seen in wine are given in Table 2. Errors for the 1 mg/l samples, employing the LL generated equations, range from 0.1 to 4.0% where as the error for LR generated values ranging from 11 to 82% (e.g. **1**). Overall, for concentrations above 20–25 mg/l, the values obtained by the two equations are similar (largest deviation is for **1** ranging from 4.0 to 0.4% at 75 mg/l). For acids in low concentration, the LL equations were used to calculate concentrations.

3.4. Food analysis

3.4.1. Sample preparation

Although the sample preparation is crucial for accurate food composition data, the main purpose of this work was the development of a multi-component measurement system. To ensure reproducibility, however, sample preparation and recovery experiments were performed. Phenolic acids are present in foods mainly as esters, acetals and glycosides. A common technique to isolate phenolics from food matrices is hydrolysis to liberate the acids followed by fractionation [1]. The latter, is a technique whereby the differences between the pK_a values of the phenolic and carboxylic proton is exploited for separation from neutral food components and larger polyphenols [10]. The pK_a of the phenolic hydrogen is around 10 whereas that of the phenolic carboxylic acid proton is between 4 and 5. For wines, the vinification process liberates the phenolic acids allowing for direct extraction of wine samples. Initial results in our laboratory indicate to a considerable loss of phenolic acids after the fractionation step. Treatment of wine with $NaHCO_3$ to isolate the acids reduces recovery by 80% relative to an untreated wine sample. However, maximum yields of acids were obtained using a simplified published procedure, where no pH adjustments were made and wine was directly extracted with ethyl acetate (3×) [15,17]. Although wine samples are commonly adjusted to pH 2 (with an aqueous HCl solution) [14], prior to extraction, our investigations indicate that there is no difference in the recovery of phenolic acids with or without pH adjustment. In addition, using sonication (instead of mechanical stirring) as the means of enhancing molecular interaction greatly reduced the formation of emulsions and eliminated a centrifuge step. Drying the extract with $MgSO_4$ did not decrease the quantities of phenolic acids recovered (as compared to an untreated sample). To assess recovery behavior, standards solutions were treated under identical extraction conditions. Recoveries obtained with this method ranged from 69 to 97%. An internal standard, 2,3,4-trihydroxybenzoic acid (**3**), was added to

Table 2
Error analysis on calibration curves for the six phenolic acids found in red wine samples analyzed

Acid[a]	Equations (LL and LR plot)	R^2	Concentration of standard solutions (mg/l)	Error from log–log plot equation (%)[b]	Error from direct linear regression (%)
1	$y = 0.9687x + 5.1937$	0.9998	75.18	0.13	1.6
	$y = 140154x - 96397$	0.9998	1.00	1.3	81.7
2	$y = 1.0113x + 4.9033$	1	46.4	0.2	0.3
	$y = 83762x - 11959$	0.9999	1.00	0.3	10.9
7	$y = 0.9917x + 5.2167$	0.9996	42.70	1.6	0.2
	$y = 162786x - 36961$	0.9998	1.00	2.3	26.2
9	$y = 0.967x + 5.4304$	0.9985	52.10	3.1	1.7
	$y = 237167x + 127887$	0.9992	1.00	4.0	35.5
11	$y = 1.0027x + 5.0999$	0.9999	42.99	2.2	2.2
	$y = 238608x + 54858$	0.9999	1.00	1.3	1.6
13	$y= 0.9978x + 5.3859$	0.9996	30.54	2.4	2.6
	$y= 242101x - 21413$	0.9998	1.00	2.0	7.8
14	$y = 0.994x + 5.3921$	0.9996	41.42	1.7	1.1
	$y = 238608x + 54858$	0.9991	1.05	0.1	17.5

[a] See Table 1 for peak identification.

[b] Concentrations obtained were from mean areas of four analyses of standard solutions. Errors obtained from $((C_{actual} - C_{calculated})/C_{actual}) \times 100$, where C is the concentration.

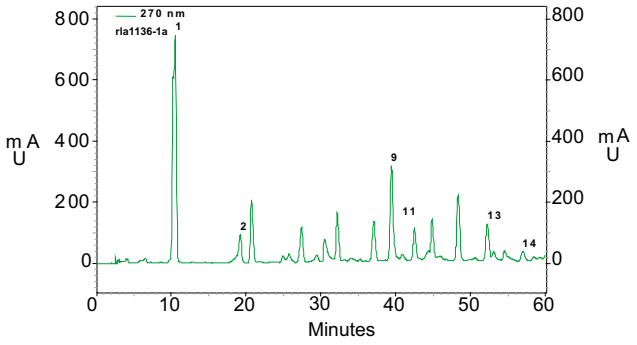


Fig. 4. Chromatogram of a Zinfandel wine sample extracted with ethyl acetate (3×) monitored at 270 nm. Labeled peaks are phenolic acids (see Table 1). Identity confirmed by UV, retention time and spiking with commercially available standard. Concentrations of phenolic acids in this sample were: **1** (34.6 mg/l), **2** (3.7), **9** (22.1), **11** (3.8), **13** (7.7), **14** (2.2).

the wine prior to extraction. Recovery of **3** was 80.2%. No single definitive sample preparation method (e.g. extraction and hydrolysis) for phenolic acids currently exists. Systematic investigations to optimize the hydrolysis and extraction steps are currently underway in our laboratory.

3.4.2. Results

Fig. 4 displays a chromatograph collected at 270 of the Zinfandel wine sample, using our optimized extraction procedure. Seven phenolic acids were observed in this wine sample. Since several absorption spectra of the phenolic acids were similar (e.g. **9** and **14**), peaks assignments were made using the retention times, spiking the wine sample with purchased standards in addition to the UV–Vis spectra of analytes to purchased standards (at expected t_R). The retention times obtained for the phenolic acids in wine samples are comparable to those obtained with the standard solution. For triplicate analyses of both standards and wine samples, the R.S.D.s ranged from 0.07 to 0.5% for the different acids ($n = 6$).

Concentrations obtained for different wine samples analyzed in our laboratory with our measurement system are listed in Table 3. Variation in the concentrations of phenolic acids from different wines is expected considering their biosynthetic origin stems from adaptation to environmental influences (e.g. time of harvest, weather, and geographic location to name a few). Reproducibility, even on the same food sample, can be difficult. Comparison to literature values is also difficult since the sample preparation and extraction conditions vary considerably. Yet, for the Bordeaux wine, the same seven phenolic acids were reported (Table 3) in previously published work [15,17]. In all cases, except for **14**, the concentrations we obtained were larger than the reported values. This might be in part due to expected variation as well as the optimized extraction procedure performed.

Due to this naturally occurring variation, and since no standard reference materials for phenolic acids exist, wine samples originating from the same wine bottle and stored under similar conditions were sent to a collaborating laboratory. The same extraction method (as indicated in Section 2.2.2), the mobile phase, gradient program and column were employed as in our laboratory. Each wine sample was repeated four times (i.e. from sample preparation—extraction to chromatographic analysis.) The inter-laboratory variation (R.S.D.) ranges from 0.9 to 8.7% for FCL and 0.6 to 18.1% for GPRMC.

The AOAC International guidelines indicate that precision may be estimated from R.S.D. $= 2C^{-0.15}$, where C is the concentration. Although the amounts of the phenolic acids vary in the different wine samples, the concentrations range from 0.3 to 80 ppm thereby allowing a reproducibility deviation of 11–16%. For both laboratories deviation is highest for ferulic acid (**14**) in the Vendange wine. GPRMC measurements for **14** range from 0.8 to 1.2 g/ml where as for FCL the range went from 0.52 to 0.64 mg/l (generated by LL equation). It is, however, not unexpected that with low concentrations, the errors are larger. R.S.D.s for all other data fall within the acceptable range (Table 4).

Wine samples were spiked with a known amount of one phenolic acid standard (as indicated in Section 2.3) for the purpose of establishing recovery. Recovery ranged from 79 to 87% (based on concentrations obtained from FCL results of spiked and non-spiked samples). These results are consistent with the internal standard recovery described above. Table 4 lists results—from the two laboratories (FCL and GMRPC)—for the three wine samples (Bordeaux, Vendange and Zinfandel). A correlation plot for the Zinfandel and Vendange wines indicates that the data sets are consistent with one another (Fig. 5). In all three cases, however, the slope is less than one, indicating measurements made at FCL are consistently lower than those made at GMPRC. The slopes

Table 3
Phenolic acid content (mg/l ± S.D.) of four red wines samples analyzed.

Wine	**1**	**2**	**7**	**9**	**11**	**13**	**14**
Zinfandel ($n = 6$)	41 ± 3.2	2.9 ± 0.2	–[a]	23 ± 2.1	3.6 ± 0.3	8.2 ± 1.2	1.8 ± 0.3
Vendange ($n = 3$)	31 ± 1.7	2.3 ± 0.1	1.9 ± 0.1	5.9 ± 0.4	3.8 ± 0.5	3.6 ± 1.1	0.54 ± 0.1
Gallo ($n = 3$)	18 ± 0.2	3.6 ± 0.1	–	6.6 ± 0.2	3.8 ± 0.3	2.6 ± 0.1	0.27 ± 0.2
Bordeaux ($n = 3$)	72 ± 2.7	5.0 ± 0.1	2.9 ± 0.2	5.4 ± 0.2	4.9 ± 0.2	2.2 ± 0.1	0.37 ± 0.1
Literature values for Bordeaux[b]	5.8–43.1	0.2–1.10	0.3–1.6	0.9–2.1	1.0–3.8	0.1–1.3	0.1–0.8

[a] Not quantifiable.
[b] Values obtained from [15,17].

Table 4
Concentrations of the seven phenolic acids found in the red wine samples

Wine	Lab	1	2	7	9	11	13	14
Zinfandel[a] ($n = 4$) (R.S.D.)	GMPRC[b]	40.5 ± 0.3 (0.6)	3.0 ± 0.1 (2.5)	9.9 ± 0.2 (4.1)	22.3 ± 0.4 (1.7)	6.4 ± 0.5 (7.6)	6.0 ± 0.1 (0.9)	2.0 ± 0.1 (5.8)
	FCL[c]	38.0 ± 2.0 (5.3)	3.0 ± 0.1 (3.5)	8.5 ± 0.2 (2.6)	19.4 ± 0.9 (4.6)	4.0 ± 0.2 (5.5)	7.3 ± 0.4 (4.8)	1.6 ± 0.1 (6.9)
Bordeaux[d] ($n = 4$) (R.S.D.)	GMPRC	75.5 ± 0.8 (1.0)	5.1 ± 0.2 (4.4)	5.4 ± 0.4 (6.7)	6.6 ± 0.6 (9.3)	7.3 ± 0.3 (3.9)	2.9 ± 0.4 (13.3)	5.5 ± 0.3 (4.7)
	FCL	70.7 ± 1.4 (2.0)	4.8 ± 0.1 (1.1)	2.9 ± 0.1 (2.0)	4.9 ± 0.1 (0.9)	5.5 ± 0.3 (5.6)	2.9 ± 0.1 (1.1)	4.7 ± 0.1 (1.4)
Vendange[e] ($n = 4$) (R.S.D.)	GMPRC	33.8 ± 1.3 (3.7)	2.8 ± 0.2 (6.0)	3.23 ± 0.3 (8.6)	18.3 ± 0.6 (3.1)	5.9 ± 0.4 (6.0)	4.0 ± 0.6 (15.1)	1.0 ± 0.2 (18.1)
	FCL	29.7 ± 2.0 (6.6)	2.4 ± 0.1 (4.7)	1.9 ± 0.1 (4.4)	13.8 ± 1.0 (7.5)	3.8 ± 0.2 (6.4)	4.2 ± 0.2 (5.0)	0.6 ± 0.1 (8.9)

Values obtained from two laboratories: Food Composition Laboratory (FCL) and Grain Marketing and Production Research Center (GMPRC).
[a] Spiked with caffeic acid (**9**).
[b] GMPRC: Grain Marketing and Production Research Center.
[c] FCL: Food Composition Laboratory.
[d] Spiked with ferulic acid (**14**).
[e] Spiked with vanillic acid (**7**).

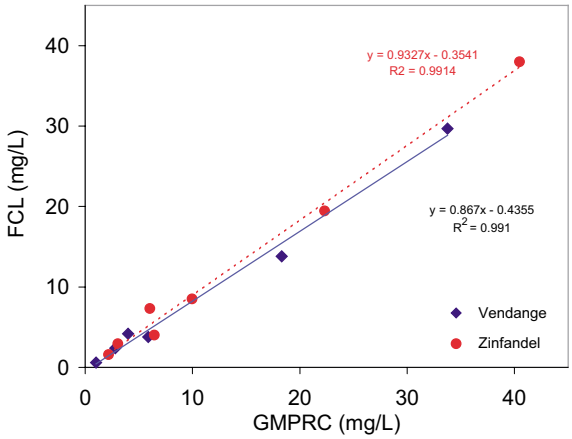


Fig. 5. Correlation plot between wine data obtained for Zinfandel and Vendange wine samples from Food Composition Laboratory (FCL) and Grain Marketing and Production Research Center (GMPRC).

for both the Bordeaux and Zinfandel wine samples approach one (0.97 and 0.93, respectively). Due to the difference in scales, the Bordeaux sample is not plotted, however, the linear regression gave $y = 0.969x - 0.862$ with a correlation coefficient of 0.9989. The Vendange samples deviate more with a slope of 0.87. All correlation data generates R^2 values above 0.99 indicating that the deviations are small.

4. Conclusion

We have developed an HPLC method for the separation and quantitation of phenolic acids expected to be prominent in commonly consumed foods. The method is based on reversed-phase column (Phenomenex Luna C_{18}-high-purity silica (150 mm × 4.6 mm, 5 μm) technology with a binary gradient system consisting of methanol and water containing 0.1% formic acid. Sixteen phenolic acids were separated. Acids were monitored at selected wavelength representing the lowest energy λ_{max} for individual acids as well as 270 nm. External calibration technique was employed and responses for the standards were linear over a large concentration range (0–100 mg/l) for all acids. Equations were generated by linear regression on raw data and on the log of our data (concentration and area). Error generated by both equations was assessed over the range of concentration. The large errors obtained for the low concentration samples (1 mg/l) were minimized by using the log plots. LODs were estimated to be 0.01–0.03 mg/l based on running 30 blanks and multiplying the standard deviation by 3 and then converting to concentration. The application of the measurement system was demonstrated by analyzing wine extracts known to contain free phenolic acids. Finally, correlation between quantitative results from two laboratories generated linear regression equations that approached unity (0.93–0.98) and with R^2 values ranging from 0.990–0.999.

References

[1] H.M. Dawes, J.B. Keene, J. Agric. Food. Chem. 47 (1999) 2398.
[2] M. Ellnain-Wojtaszek, Z. Kurczynski, J. Kasprzak, Acta Polonae Pharm. Drug Res. 58 (2001) 205.
[3] S.H. Häkkinen, S.O. Kärenlampi, I.M. Heinonen, H.M. Mykkänen, A. Riitta, J. Sci. Food Agric. 77 (1998) 543.
[4] K. Herrmann, Crit. Rev. Food Sci. Nutr. 28 (1989) 315.
[5] R.A. Jacob, B.J. Burri, Am. J. Clin. Nutr. 63 (1996) 985S.
[6] G.L. Long, J.D. Winefornder, Anal. Chem. 55 (1983) 712 A.
[7] J.A. Maga, CRC Crit. Rev. Food Sci. Nutr. 10 (1978) 323.
[8] N.J. Miller-Ihli, T.C. O'Haver, J.M. Harnley, Spectrochim. Acta 39 (1984) 1603.
[9] L.W. Morton, R.A.-A. Caccetta, I.B. Puddey, K.D. Croft, Clin. Exp. Pharmacol. Physiol. 27 (2000) 152.
[10] J. Oszmianski, T. Ramos, M. Bourzeix, Am. J. Enol. Vitic. 39 (1988) 259.
[11] G. Paganga, N. Miller, C.A. Rice-Evans, Free Rad. Res. 30 (1999) 153.
[12] C.A. Rice-Evans, N.J. Miller, G. Paganga, Free Rad. Biol. Med. 20 (1996) 933.
[13] K. Robards, P.D. Prenzler, G. Tucker, P. Swatsitiang, W. Glover, Food Chem. 66 (1999) 400.
[14] R.J. Robbins, J. Agric. Food Chem. 51 (2003) 2866.
[15] M.A. Rodríguez-Delgado, S. Malovaná, J.P. Pérez, T. Borges, F.J. García Montelongo, J. Chromatogr. A 912 (2001) 249.
[16] H. Sakakibara, Y. Honda, S. Nakagawa, H. Ashida, K. Kanazawa, J. Agric. Food Chem. 51 (2003) 571.
[17] M.-H. Salagoïty-Auguste, A. Bertrand, J. Sci. Food Agric. 35 (1984) 1241.
[18] F. Shahidi, M. Nacsk, Food Phenolics: Sources, Chemistry, Effects and Application, Technomic Publ. Lancaster, PA, 1995.
[19] F.A. Tomás-Barberán, J.C. Espín, J. Sci. Food Agric. 81 (2001) 853.
[20] G. Williamson, G.W. Plumb, M.T. Garcia-Conesa, Plant Polyphenols 2: Chemistry, Biology, Pharmacology, Ecology, Plenum Press, New York, p. 483, 1999.
[21] H. Wu, T. Haig, J. Prately, D. Lemerle, M. An, J. Agric. Food Chem. 48 (2000) 5321.
[22] A. Ziaková, E. Brandsteterová, J. Liq. Chromatogr. Rel. Technol. 26 (2003) 443.