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A rapid, one step preparation for measuring selected free plus SO_2-bound wine carbonyls by HPLC-DAD/MS

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ABSTRACT

Carbonyl compounds are produced during fermentation and chemical oxidation during wine making and aging, and they are important to wine flavor and color stability. Since wine also contains these compounds as α-hydroxysulfonates as a result of their reaction with sulfur dioxide, an alkaline pretreatment requiring oxygen exclusion has been used to release these bound carbonyls for analysis. By modifying the method to hydrolyze the hydroxysulfonates with heating and acid in the presence of 2,4-dinitrophenylhydrazine (DNPH), the carbonyl compounds are simultaneously and quickly released and derivatized, resulting in a simpler and more rapid method. In addition, the method avoids air exclusion complications during hydrolysis by the addition of sulfur dioxide. The method was optimized for temperature, reaction time, and the concentrations of DNPH, sulfur dioxide and acid. The hydrazones were shown to be stable for 10 h, adequate time for chromatographic analysis by HPLC-DAD/MS. This method is demonstrated for 2-ketoglutaric acid, pyruvic acid, acetoin and acetaldehyde, wine carbonyls of very different reactivities, and it offers good specificity, high recovery and low limits of detection. This new rapid, simple method is demonstrated for the measurement of carbonyl compounds in a range of wines of different ages and grape varieties.

1. Introduction

Carbonyl compounds are common byproducts of many metabolic processes and chemical oxidation of major wine components during wine making and aging [1–3]. They are frequently cited as volatile organic compounds in wines that can play a major role in the aroma character of fermented beverages [4]. In some cases, the levels of these compounds in beverages can be an indicator of deterioration caused by pasteurization, storage or even an indicator of contamination [5]. To date, well-characterized carbonyl substances found in the wine include acetaldehyde, pyruvic acid, 2-ketoglutaric acid, glyceraldehyde, formaldehyde, acetoin, glucuronic acid, sugars and diacetyl [6,7]. Among these carbonyls, the levels of acetaldehyde, pyruvic acid and acetoin are quite high, with reported levels as high as 490, 460 and 350 mg L^{-1} respectively, while the others have been observed at much lower levels or have low reactivity [1,8]. In addition, acetaldehyde, pyruvic acid, and glyceraldehyde are key wine oxidation products.

Depending on their concentration and structure, these carbonyls can contribute pleasant or undesirable notes to wine and other fermented beverages [4,9]. For instance, the saturated short-chain aldehydes significantly affect overall flavor, contributing notes such as nutty, bruised apples, herbaceous, grassy, green, fatty, fruity and pungent [10], while the significance of acetaldehyde to wine aroma is questionable, as no correlation was found between this oxidation product and oxidation flavors in young white wines [11].

Carbonyls are also known to take part in important wine aging reactions, with potential benefits to the color stabilization of red wines. Aldehydes may take part in the formation of ethyl-linked compounds, which are very important for red wine color development [12,13]. Acetaldehyde, the main secondary product of oxygen reduction, can initiate reactions between anthocyanins and flavanols to generate a product with an ethyl bond, [12,14,15]. Direct reactions of acetaldehyde with malvidin-3-glucoside produce vitisin B, an important color-stabilized product [16]. Ketoacids may be also important for wine color stabilization, and pyruvic acid reacts with malvidin-3-monoglucoside to form pyranoanthocyanins. This formation results from cyclisation between C-4 and the hydroxyl group at C-5 of the original flavylium moiety with the double bond of the enolic form of pyruvic acid, followed by dehydration and rearomatisation steps. These newly generated compounds resist color changes from pH shifts and sulfur dioxide bleaching [17]. Aside from the effects on color, aldehydes may also improve wine taste and structure; acetaldehyde plays an important role in polymerization

Abbreviations: CS, Cabernet Sauvignon; CV, coefficient of variation; DNPH, 2,4-dinitrophenylhydrazine; HPLC, high performance liquid chromatography

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and precipitation of water-soluble proanthocyanidins, resulting in less astringent wines [18,19].

For analyzing carbonyls, there are numerous techniques available including non-specific methods such as non-quantitative thin layer and paper chromatography methods, low sensitivity methods based on colorimetric procedures and distillation or reaction with bisulfite [2,20–22]; enzymatic redox reaction methods which are used for single compounds [23,24]; and gas chromatography methods [25,26]. Alternatively, liquid chromatography methods [26], with equivalent accuracy, sensitivity and specificity have been developed which were based on the reaction with hydrazines, such as 2,4-dinitrophenylhydrazine (DNPH), to form stable hydrazones. Unfortunately, most of these methods are not applicable to wine because they do not account for sulfite-bound forms. At wine pH (between 3 and 4) sulfites are mainly present in the bisulfite ion form (HSO_3^-), which binds reversibly to carbonyls [27,28], to form α-hydroxysulfonates, decreasing the apparent amount of carbonyls [21,29,30]. Thus a treatment to dissociate sulfite-bound carbonyls is most important for a quantitative method for the analysis of total (i.e., free and sulfite-bound) carbonyl compounds in wine.

The most common method to release the α-hydroxysulfonates involves alkaline hydrolysis. The α-hydroxysulfonates are formed by the reaction of carbonyls with bisulfite, but when the pH of solution is below 1 (pKa: 1.85) or above pH 8 (pKa 2: 7.2) [31], sulfites are primarily present in the forms (SO_2 or SO_3^{2-}, respectively), forms that do not react with carbonyls, so the adduct does not re-form once the bond is broken. However, strong alkaline conditions generally accelerate oxidation, thus potentially resulting in the formation of additional carbonyl compounds, [7,32,33] so anaerobic handling is necessary, difficult for a large number of samples. The alkaline hydrolysis is followed by acidification and a derivatization step typically with 2,4-dinitrophenylhydrazine (DNPH) [7]. A recently-reported improved method by Jackowetz eliminated the need for anaerobic sample handling through addition of EDTA to chelate with metals. This prevents acetaldehyde formation from ethanol oxidation, but following alkaline hydrolysis, a lengthy 30 h was required for hydrazone formation. [6]. While this long reaction time was required principally for the derivatization of glucose and galacturonic acid, not tested here, the Jackowetz method still requires two steps, alkaline hydrolysis followed by acidification and derivatization.

To simplify the sample preparation protocol, our approach was to evaluate the use of acid hydrolysis of the hydroxysulfonates, and to also test antioxidants to avoid the need for air exclusion during sample handling. The combination of these was evaluated with sample warming to accelerate the process. This new procedure would provide one result for each carbonyl compound, totaling the free and SO_2-bound forms, and do so quickly and simply.

2. Materials and methods

2.1. Reagents and wine samples

DNPH (30% water, m/m) was obtained from Alfa Aesar (Ward Hill, MA, USA) and was purified by recrystallization from acetonitrile. Acetaldehyde, 2-ketoglutaric acid, pyruvic acid and acetoin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The corresponding DNPH hydrazone standards were prepared as described previously and recrystallized from acetonitrile [34] All solutions were prepared with Milli-Q water from Millipore (Bedford, MA, USA), and other chemicals and solvents were HPLC grade and were obtained from Fisher (Fairlawn, NJ, USA) or Sigma-Aldrich (St. Louis, MO, USA). Model wine solutions consisted of 12% ethanol (v/v) in (+)-tartaric acid solution (5 g L^{-1}), adjusted to pH 3.6 with sodium hydroxide (5 N).

Red and white wine samples used in the research were either donated to, or produced by the Department of Viticulture and Enology at the University of California, Davis (Supplemental Table 1), and were analyzed shortly after opening. Solutions of varying concentrations of sulfur dioxide, freshly prepared from potassium metabisulfite (57% SO_2, m/m, although water content of the salt was not rigorously controlled), were used for method development and validation.

2.2. Instrumentation and carbonyl compounds detection

For identification and confirmation of carbonyls in wine samples, a liquid chromatographic system (HP 1100 series, Agilent Technologies, Wilmington, DE) coupled to a mass detector (HP 1100 MSD series, Agilent Technologies) equipped with an ESI interface was used. UV detection was obtained by diode array (DAD), monitoring at 365 nm. In the chromatographic system, a ZORBAX Rapid Resolution HT, SB-C18 column (1.8 μm, 4.6 × 100 mm^2, from Agilent Technologies) was used for separation. The LC system consisted of binary pumps, a variable volume autosampler and a thermostated column compartment.

The chromatographic conditions used were based on a previous method [7]: sample injection volume, 15 μL; flow rate, 0.75 mL min^{-1}; column temperature, 35 °C; mobile-phase solvents, (A) 0.5% (v/v) formic acid in water and (B) acetonitrile; gradient elution protocol (v/v), 35–60% B (8 min), 60–90% B (13 min), 90–95% B (15 min, 2 min hold), 95–35% B (16 min, 4 min hold), total run time, 20 min. For mass spectrometry, the negative ion mode was used with the following conditions: capillary temperature, 350 °C; sheath gas (N_2) flow at 80 arbitrary units and auxiliary gas (N_2) flow at 30 arbitrary units. Mass detection was performed over the range 120–2000 m/z.

The identification of the observed carbonyls was based on their retention time compared with standards as well as mass spectral data for confirmation. Data analysis and peak integration was carried out using the Agilent Chemstation (A 09.03) software package.

2.3. Derivatization procedure and variables

Derivatizations were conducted manually in 2.0 mL glass vials (15 mm × 85 mm, Fisher) with Teflon lined caps. Sample aliquots (100 μL) were dispensed to the vial, followed by 20 μL of freshly prepared sulfur dioxide solution (0, 840, 1120, 1400, 3360 mg L^{-1}), and then 20 μL of sulfuric acid (0%, 5%, 15%, 25%) (v/v) was added followed by 140 μL of the DNPH reagent (2, 4, 6, 8 g L^{-1}). The 8 g L^{-1} DNPH solution was obtained by warming and ultrasonic treatment. After mixing, the added sulfur dioxide in the reaction solutions was 0, 60, 80, 100 or 240 mg L^{-1}, sulfuric acid was 0, 0.36%, 1.1% or 1.8% (v/v) and DNPH was 0.14, 0.29, 0.43 or 0.57 g L^{-1}. The solutions were allowed to react for 5, 10, 15, 20, or 25 min at 45, 65, or 85 °C and then promptly cooled to room temperature. As each factor was tested, other factors remained constant, so only one factor was varied at a time during the optimization of each variable. To avoid hydrazone crystallization during chromatography, samples were diluted 1:1 in mobile phase A following DNPH derivatization, and filtered through 0.45 μm polytetrafluoroethylene (PTFE), 13 mm, syringe tip filters (Arcodisc TM, Ann Arbor, MI, USA) into 2 mL HPLC vials and sealed with PTFE crimp caps. Each completely derivatized wine sample was analyzed by HPLC-DAD/MS immediately. To compare the effect of acid hydrolysis on release of free carbonyls, the traditional alkali hydrolysis method [7] was used as a control.

With the optimized sample preparation procedure, completely derivatized wine samples were stored for 3, 10, or 24 h at room temperature before injection into the LC, to check the effect of prolonged storage of derivatized wine samples at ambient temperature.

2.4. Assay validation

Linearity/working range. To check the linearity of the developed method, an external calibration curve for the hydrazone standards in model wine solution, in the range of 1.5–600 mg L^{-1} for 2-ketoglutaric and acetoin, 1–400 mg L^{-1} for pyruvic acid and acetaldehyde, was run in triplicate and the respective peak area signal responses were recorded.

These concentration ranges were based on the reported levels in wine, and allowed for the determination of all the analytes in a single chromatographic run. The response curve was determined by calculating the slope of the data by linear regression and the correlation coefficient (r^2) for the quality of the line, using the Microsoft Excel 2013 software package.

2.5. Extraction recovery

The extraction recoveries were assayed by analyzing model wine spiked with 2-ketoglutaric acid, pyruvic acid, acetoin and acetaldehyde at the concentrations of 5, 20 and 50 mg L^{-1} ($n=6$). This parameter was determined using the equation [22,23,25]

$$\text{Recovery}(\%) = \frac{\text{Average concentration}}{\text{Theoretical concentration}} \times 100 \quad (1)$$

Precision: Spiked model wine of 2-ketoglutaric acid, pyruvic acid, acetoin and acetaldehyde at three concentrations (5, 20 and 50 mg L^{-1}) were assayed to determine precision ($n=6$) on the same day. The coefficient of variation (CV) was calculated by dividing the standard deviation of the concentration of each analyte by the average concentration and multiplying by 100 [20,22,23]

$$\text{CV}(\%) = \frac{\text{Standard deviation}}{\text{Average concentration}} \times 100 \quad (2)$$

2.6. Method application

The method was applied to the determination of the four carbonyl compounds in five white and seven red wines, including *Cabernet Sauvignon*, *Merlot*, *Sauvignon blanc*, *Albarino* and *Verdelho* wines, described in Table 4 and Supporting information.

3. Results and discussion

3.1. Acid hydrolysis of sulfite-bound carbonyls and derivatization with DNPH

The traditional alkaline hydrolysis of sulfite-bound carbonyl compounds can result in accelerated air-induced oxidation, inadvertently producing additional carbonyls in the process [7]. To avoid this oxidation, at least 30 min of degassing is needed to deoxygenate the reagent and reaction solutions with nitrogen gas (N_2). One means to avoid this oxidation is to add a metal chelator [6]. Here, acid hydrolysis was used to replace alkaline hydrolysis, using an elevated reaction temperature, 65 °C to reduce reaction time. However, even under acidic conditions, some oxidation can occur in air, so an antioxidant, SO_2, was used to scavenge hydrogen peroxide and to reverse any quinone formation.

Either high or low pH conditions would be useful to dissociate the hydroxysulfonates. So, our approach was to investigate acid hydrolysis, and at the same time avoid an oxygen exclusion protocol by adding an antioxidant. Sulfur dioxide was tested, and while counter-intuitive as the treatment is designed to cleave hydroxysulfonates, it seemed feasible because the low pH leaves SO_2 in a non-nucleophilic form.

Effect of acid concentration on bound acetaldehyde release. The effect of low pH on acetaldehyde release during the assay was examined in CS wine produced in 2004 by University of California, Davis (UCD) (Fig. 1), which was identified as having the highest level of SO_2 bound carbonyls [6]. Using a SO_2 concentration of 80 mg L^{-1}, at 65 °C, and 0.57 g L^{-1} DNPH, sulfuric acid concentrations of (v/v) 0, 0.36%, 1.1% and 1.8% were tested. The selected sulfuric acid concentrations were based on previous investigations [6,7]. To ensure that the increased detection of acetaldehyde had nothing to do with the oxidation of wine at low pH, an oxygen exclusion step was tested as well. N_2-sparged reagents were used and the headspace was blanketed with N_2 during sample hydrolysis. When the sulfuric acid concentration was 0.36%, a rapid increase in the concentration of derivatized acetaldehyde (from 3 mg L^{-1} to more than 9 mg L^{-1} in less than 10 min) was observed compared to the reaction to which no acid was added. At 1.1% sulfuric acid, the acetaldehyde released after 10 min increased by 1.2 mg L^{-1} compared with 0.36%. There was no significant difference ($p<0.05$) between 1.1% and 1.8% sulfuric acid. However, at 15 min reaction time, another 1.2 mg L^{-1} acetaldehyde was released at 1.8% H_2SO_4, but longer times had no effect. Therefore, the 15 min reaction time with 1.8% acid was chosen to ensure all bound acetaldehyde was released. Method validation (below) demonstrates excellent recovery, confirming the adequacy of this level of acid. Slight increases beyond 25 min suggested a possible loss of SO_2 and consequent oxidation (data not shown). A comparison of methods also demonstrated that there was no statistically significant difference ($p<0.05$) between samples analyzed by acid hydrolysis versus the traditional alkali hydrolysis method used by Elias et al. [7].

Effect of SO_2 on oxidation protection. To eliminate any oxidation of wine induced by ambient air, extra sulfur dioxide was used to protect the wine. This antioxidant reagent may seem counter-intuitive as the treatment is used to break down sulfonates. Sulfur dioxide does not react directly with oxygen but with its reduced forms such as hydrogen peroxide, [35–38] So, SO_2 can inhibit aldehyde formation by scavenging hydrogen peroxide, preventing the formation of hydroxyl radicals via the Fenton reaction, the source of aldehydes in wine oxidation [33].

To validate the antioxidant effect of SO_2 at high temperature, the concentration of acetaldehyde was measured in a red wine (CS, 2004, UCD) containing or excluding extra SO_2, and permitting or preventing air exposure with nitrogen. (Fig. 2). This process used 1.8% sulfuric acid, at 65 °C, and 0.57 g L^{-1} DNPH solution, with additions of SO_2 that would provide concentrations of 0, 60, 80,

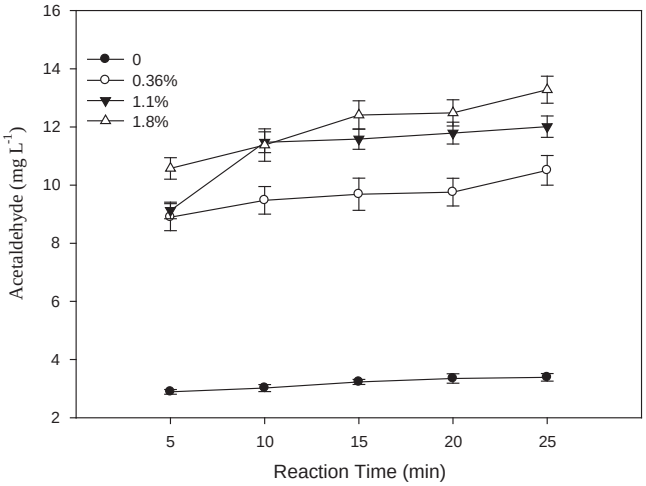

Fig. 1. Levels of acetaldehyde hydrazone observed in a 2004 Cabernet Sauvignon wine at different concentrations of H_2SO_4. The reaction conditions are 80 mg L^{-1} SO_2, and 0.57 g L^{-1} DNPH, at 65 °C.The error bars are standard deviation.

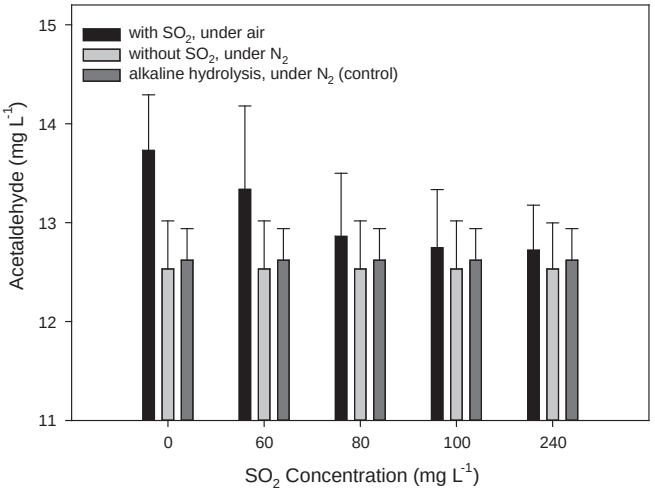

Fig. 2. Effect of added SO_2 on the level of acetaldehyde observed in a 2004 Cabernet Sauvignon wine, compared to a traditional alkaline hydrolysis test method. [7] The reaction condition is 1.8% sulfuric acid, 0.57 g L^{-1} DNPH, at 65 °C. The error bars are standard deviation.

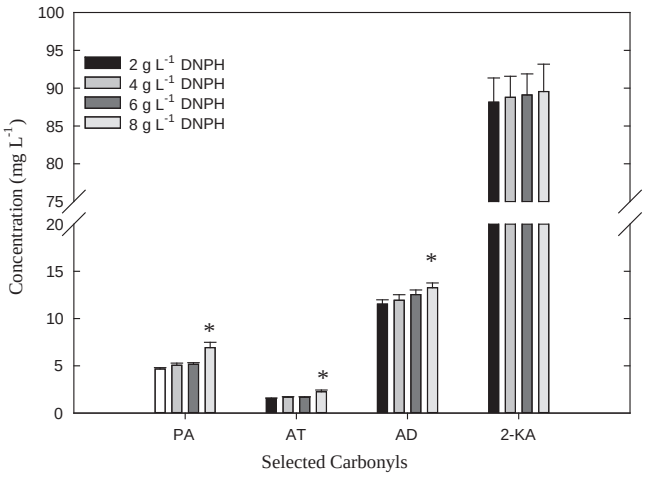

Fig. 3. Effect of DNPH concentration on derivatization of selected carbonyls in 2004 Cabernet Sauvignon wine (2004). The error bars are standard deviation. * indicate statistical differences ($p < 0.05$). The reaction condition is 1.8% sulfuric acid, 80 mg L^{-1} SO_2, at 65 °C. Abbreviations: PA: pyruvic acid; AT: acetoin; AD: acetaldehyde; 2-KA: 2-ketoglutaric acid.

100 and 240 mg L^{-1} in the reactions solutions. In reactions without added SO_2, a significant ($p < 0.05$) increase in acetaldehyde concentration was observed. But with added SO_2, a significantly decrease ($p < 0.05$) in acetaldehyde was observed. At 80 mg L^{-1} SO_2 acetaldehyde levels were significantly ($p < 0.05$) lower than the SO_2 free sample, but there was almost no difference between 80 mg L^{-1} and 240 mg L^{-1}, those being similar to the oxygen free control. Thus the 80 mg L^{-1} SO_2 treatment was adequate to prevent the formation of additional acetaldehyde. As the conventional wisdom is that the bisulfite (HSO_3^-) form is what reacts with H_2O_2, [39] this result suggests that the small fraction of the bisulfite (HSO_3^-) form present under these low pH conditions (pH = 1.1) was adequate to prevent wine oxidation induced by higher temperature.

Previous results by Lea et al. showed that levels up to 250 mg L^{-1} did not impact derivatization efficiency with DNPH [24], albeit under basic conditions. The potential interference under acidic conditions was then tested by comparing an analysis under nitrogen, with and without SO_2. The level of acetaldehyde detected was 12.4 ± 0.5 mg L^{-1} with 240 mg L^{-1} SO_2 and 12.5 ± 0.5 mg L^{-1} without extra SO_2. Thus under these conditions, the added SO_2 does not interfere with the release of the bound carbonyl compounds. at least up to 240 mg L^{-1}.

Effect of DNPH addition on derivatization. After defining the optimized amount of sulfuric acid and SO_2, new tests were conducted to compare concentrations of DNPH. Using a SO_2 level of 80 mg L^{-1}, a reaction temperature of 65 °C, and 1.8% sulfuric acid, DNPH concentrations of 0.14, 0.29, 0.43 and 0.57 g L^{-1}, were compared for the response of the hydrazones of 2-ketoglutaric acid, pyruvic acid, acetoin and acetaldehyde. Fig. 3 shows levels of hydrazone products observed.

An increasing trend was observed as the DNPH solution increased, with significantly higher responses ($p < 0.05$) for pyruvic acid, acetoin and acetaldehyde at the highest level, so that concentration was thus selected for the optimized procedure. While this amount of reagent, 0.28 mmol, is adequate for typical table wines, samples with high concentrations (see linear ranges, Table 1) would require additional reagent.

Effect of reaction temperature. The temperature was tested at 45 °C, 65 °C, and 85 °C. Since elevated temperatures were expected to cause loss of SO_2 via evaporation, and thus lead to oxidation of ethanol to acetaldehyde, it was important to study these reactions under air versus inert gas. Using a SO_2 concentration of 80 mg L^{-1}, 1.8% acid and 0.57 g L^{-1} DNPH solution, the temperatures were

Table 1
Linearity and limits of detection of carbonyl analytes based on triplicates at each level tested (in model wine).

Compound	Linear equation	R^2	Linear range (mg L^{-1})	LOD (μg L^{-1})
2-Ketoglutaric acid	$y = 47.743x - 13.893$	0.99960	0.67–269.54	7.5
Pyruvic acid	$y = 39.987x - 8.0587$	0.99950	0.33–131.84	6.3
Acetoin	$y = 45.694x - 43.47$	0.99930	0.50–197.75	4.4
Acetaldehyde	$y = 54.074x - 3.8835$	0.99990	0.2–78.92	5.1

varied and data is shown in Fig. 4. The results show no statistically significant ($p < 0.05$) difference between samples with and without added SO_2 analyzed at 45 °C (20 min reaction) and 65 °C (15 min reaction), so the protection from air oxidation was adequate with SO_2. However, the levels of acetaldehyde at 45 °C were much lower than 65 °C even with more reaction time, signaling incomplete hydrolysis of bisulfite adducts under the 45 °C condition. On the other hand, at 85 °C the same amount of acetaldehyde was observed under N_2 compared to the reaction at 65 °C, indicating this was the maximum amount that could be recovered from the wine. However, the reaction under air had increased levels of acetaldehyde, suggesting volatile losses of SO_2, leading to sample oxidation. Consequently, the 65 °C condition was selected for the optimized conditions.

Carbonyl derivatization kinetics and sample stability at room temperature. The effect of reaction time, up to 20 min, on the derivatization efficiency of carbonyls with DNPH at 65 °C and the subsequent stability of the hydrazone solutions at room temperature (up to 24 h) were evaluated with CS, 2004 UCD. Fig. 5 shows that a stable derivatization yield was reached for all selected carbonyls at 15 min at 65 °C, with no statistically different result at 20 min. 2-Ketoglutaric acid was the slowest of the reactants with differences between 10 and 15 min. Tracking the stability of these solutions by repeated analysis, all four carbonyls were stable at room temperature throughout 10 h of storage, but, a marked increase in acetaldehyde and 2-ketoglutaric acid was observed if the DNPH derivatized wine samples were allowed to stand for 24 h or more at room temperature. It is not clear why additional product would be observed on standing, but perhaps the residual

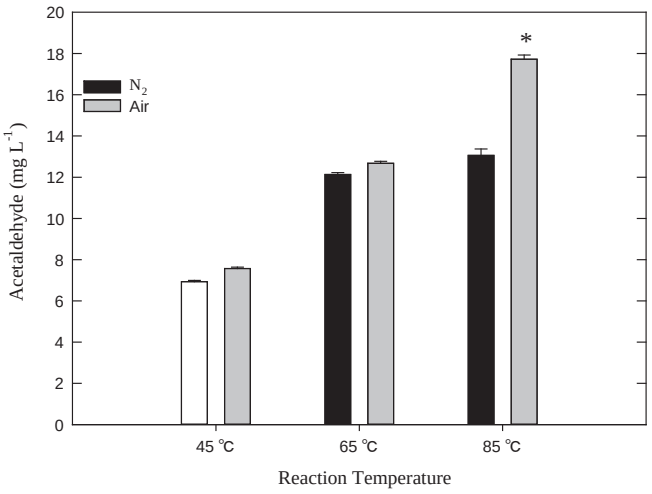

Fig. 4. Acetaldehyde levels using acid under air with SO_2 at different temperatures, compared with N_2 blanketing. The error bars are standard deviation. * indicate statistical differences ($p < 0.05$). Considering reactions at different temperature need different times, lower temperature will need longer time. So at 45 °C, the reaction time is 20 min; at 65 °C, and 85 °C the reaction time is 15 min. The reaction conditions are 1.8% sulfuric acid, 0.57 g L^{-1} DNPH, and 80 mg L^{-1} SO_2.

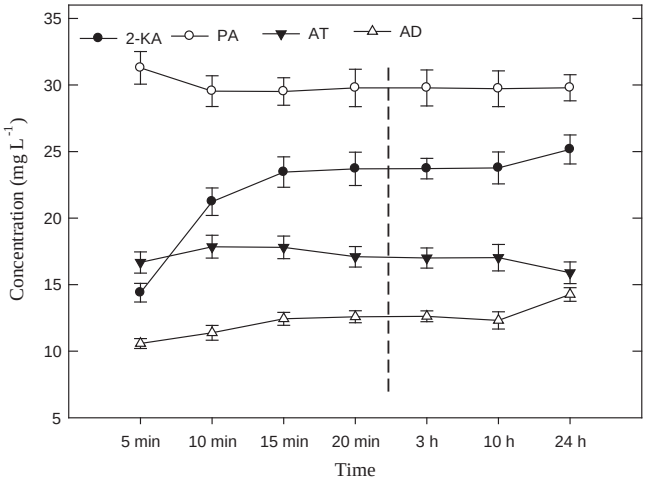

Fig. 5. Yield over different reaction times at 65 °C of four DNPH-carbonyl derivatives (5–20 min) and subsequent room temperature storage (3–24 h) of those derivatized wine samples. The error bars are standard deviation. Abbreviations: 2-KA: 2-ketoglutaric acid; PA: pyruvic acid; AT: acetoin; AD: acetaldehyde.

SO_2 is lost from the samples after 24 h and some oxidation can occur. This demonstrates the need for timely sample analysis. As the HPLC run time is 20 min, it is best to prepare no more than 30 wine samples at a time, immediately followed by chromatographic analysis in order to complete the procedure before sample instability is encountered.

Optimized procedure: Sample aliquots (100 μL) were dispensed to a vial, followed by 20 μL of freshly prepared 1120 mg L^{-1} sulfur dioxide solution, then 20 μL of 25% sulfuric acid (v/v) was added, followed by 140 μL of the 8 g L^{-1} DNPH reagent. After mixing, the added sulfur dioxide in the reaction solutions was 80 mg L^{-1}, sulfuric acid was 1.8%, and DNPH was 0.57 g L^{-1}. The solution was allowed to react for 15 min at 65 °C and then promptly cooled to room temperature. Completely derivatized wine samples were analyzed by HPLC within 10 h, storing samples at room temperature.

3.2. Chromatography

Because of the range of the hydrazone polarities, a suitable gradient of the mobile phase must be selected. A recent study [40] suggests that the water/acetonitrile mixture would be the optimal solvent for the separation of all four analytes by providing better sensitivity. To avoid hydrazone crystallization during injection and chromatography, 35% (v/v) acetonitrile was selected as the initial composition of the mobile phase. Under the gradient described above, a satisfactory chromatographic separation was achieved, with all compounds eluting in 20 min (Fig. 6). Peaks 1–4 correspond to 2-ketoglutaric acid, pyruvic acid, acetoin, and acetaldehyde, respectively, while peak H is the unreacted DNPH. Peaks were quantified by absorbance at 365 nm and analyte peaks were checked for purity by MS analysis (Fig. 6). Some of the unlabeled peaks in the chromatograms may represent other carbonyl substances that were not identified as part of this study.

3.3. Method testing

As a check on the linearity of the developed method, a series of multi-compound solutions in model wine were prepared. Analyis of these solutions using the optimized method demonstrates that good linearity of the detector response over the concentration ranges used (Table 1). For each of the compounds analyzed, the correlation coefficient (r^2) of the response was higher than 0.9993. Good detection limits were noted, ranging from 4.4 to 7.5 μg L^{-1} for all analytes in model wine, and from 0.02 to 0.2 mg L^{-1} (Table 3) in merlot wine, below the reported concentrations of most aldehydes and ketones in wine. Minimum detectable concentrations of analytes were determined as the lowest concentration of the hydrazones that yielded chromatographic peaks with signal-to-noise ratios of 3. The LOD's for other particular wine would vary depending on interferences in that wine.

Extraction recoveries were evaluated in model wine spiked at 5, 20 and 50 mg L^{-1} of each of the carbonyl standards. Recovery was determined from the mean of the triplicate experiments, applying Eq. (1). A blank revealed 2.1 mg L^{-1} acetaldehyde, attributed to impurities in the ethanol used to prepare the model wine solution, but none of other three carbonyls were observed. As shown in Table 2, the recovery values for 2-ketoglutaric acid, pyruvic acid, acetoin and acetaldehyde ranged between 98% and 98.8%, 98.7% and 99.6%, 98.2% and 98.9 and 98.7% and 99.8%, respectively, with CV's below 6% and generally 2–4%. The method thus exhibits very good recovery from low to high concentrations in model wine solutions.

Further, selected carbonyls were spiked into merlot wines (2012) to evaluate analyte recovery in actual sample matrices (Table 3). Four samples of the same Merlot wine treated with increasing levels of SO_2 (< 20–200 mg/L) were spiked using 16–20 mg/L of each analyte. The results showed that the percent recoveries of acetaldehyde ranged from 92% to 102%, while the recovery values of other three carbonyls were better, ranging from 97% to 99%. Varying levels of recovery for acetaldehyde in wine has been previously observed in the range of 88–99% [6], highlighting the issue that acetaldehyde is relatively unstable and reactive. Analysis of the data revealed no significant relationship between recovery of acetaldehyde and SO_2 concentration in wine.

3.4. Demonstration of the method

The method was applied to the determination of carbonyl compounds in twelve wines, including seven reds and five whites (Table 4). There were clear differences in the levels of these compounds between red and white wines, and among different vintages. In general, the concentration of acetaldehyde in white wines was much higher, 30–70 mg L^{-1}, than in reds, likely due to the lack of flavonoids that would be able to react with it. It was interesting to note a very low concentration of acetaldehyde in aged red Bordeaux wines from the 1960s, between 4–6 mg L^{-1},

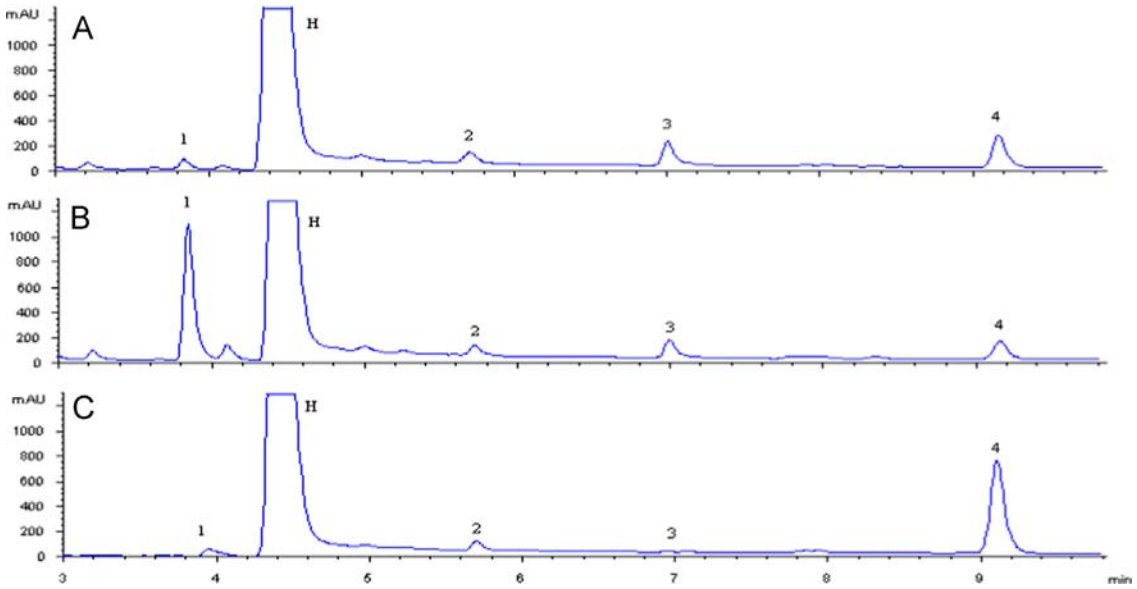

Fig. 6. HPLC/DAD chromatograms of (A) aged red wine (Cabernet Sauvignon, Woodbridge Winery, 2007; Acampo, CA), (B) young red wine (Merlot, Gallo, 2012; Sonoma Valley, CA), and (C) aged white wine (Sauvignon Blanc, University of California, 2010; Davis, CA). The observed peaks (and MS data) were 2-ketoglutaric acid (1, m/z 325), pyruvic acid (2, m/z 267), acetoin (3, m/z 267), and acetaldehyde (4, m/z 223). Peak H is the excess, unreacted DNPH reagent.

Table 2
Recovery and precision values for four selected carbonyls in model wine, $n=6$.

Compound	Spiked amount ($mg\ L^{-1}$)	Measured amount ($mg\ L^{-1}$)	Recovery (%)	SD	CV (%)
2-Ketoglutaric acid	5	4.94	98.8	0.22	4.45
	20	19.60	98	0.69	3.52
	50	49.10	98.2	1.12	2.28
Pyruvic acid	5	4.98	99.6	0.19	3.82
	20	19.84	99.2	0.67	3.38
	50	49.35	98.7	1.19	2.41
Acetoin	5	4.93	98.6	0.29	5.88
	20	19.78	98.9	0.64	3.24
	50	49.10	98.2	1.56	3.18
Acetaldehyde	5	4.91	98.2	0.2	4.07
	20	19.96	99.8	0.56	2.81
	50	49.35	98.7	1.56	3.16

Table 3
Recovery values for selected carbonyls in merlot wine (2012) containing varying levels of SO_2, $n=6$.

Compound	Spiked amount ($mg\ L^{-1}$)	Recovery (%, m/m)	LOD ($mg\ L^{-1}$)
2-Ketoglutaric acid	20	97–98	0.5
Pyruvic acid	18	98–99	0.05
Acetoin	20	97–98	0.1
Acetaldehyde	16	92–102	0.08

The initial merlot wine contained very low level of SO_2 ($<20\ mg\ L^{-1}$), and three treated samples of the same wine contained increasing levels of SO_2 for testing at $<20\ mg\ L^{-1}$, $80\ mg\ L^{-1}$, $150\ mg\ L^{-1}$ and $200\ mg\ L^{-1}$. Each level of SO_2 was spiked with the four carbonyls.

much lower than the younger red wines sourced from recent vintages at UC Davis, those having 10–30 $mg\ L^{-1}$. Overall, these levels are comparable to those previously reported [11]. The concentration of pyruvic acid in the wines paralleled acetaldehyde, suggesting a similar route of production, but 2-ketoglutaric acid and acetoin had different proportions, so these compounds do not appear to have origins in the same pathway.

Table 4
Survey of targeted carbonyls in wine samples.

Year	Variety	2-Ketoglutaric acid ($mg\ L^{-1}$)	Pyruvic acid ($mg\ L^{-1}$)	Acetoin ($mg\ L^{-1}$)	Acetaldehyde ($mg\ L^{-1}$)
1962	RB	26.50 ± 0.79	2.20 ± 0.22	1.05 ± 0.04	4.20 ± 0.13
1963	RB	27.08 ± 0.79	3.01 ± 0.25	1.54 ± 0.04	4.10 ± 0.26
1964	RB	27.79 ± 0.98	2.82 ± 0.14	4.28 ± 0.15	5.59 ± 0.17
1964	RB	21.87 ± 0.69	3.73 ± 0.40	2.94 ± 0.08	6.24 ± 0.20
2004	CS	90.03 ± 3.16	6.73 ± 0.29	1.76 ± 0.06	12.53 ± 0.45
2007	CS	13.64 ± 0.32	18.82 ± 0.46	30.32 ± 0.71	24.04 ± 0.56
2012	Merlot	195.38 ± 1.77	15.52 ± 0.72	23.40 ± 0.54	14.55 ± 0.46
2013	Albarino	13.93 ± 0.66	12.12 ± 0.23	2.79 ± 0.17	51.06 ± 0.60
2009	Verdelho	12.27 ± 0.34	12.37 ± 0.16	5.30 ± 0.07	77.49 ± 1.85
2011	SB	13.36 ± 0.63	12.86 ± 0.15	1.69 ± 0.14	37.21 ± 0.66
2010	SB	13.68 ± 0.32	12.14 ± 1.24	1.12 ± 0.06	71.02 ± 1.00
2013	WB	42.67 ± 1.06	24.13 ± 0.67	3.19 ± 0.26	32.54 ± 0.70

Abbreviations: RB: red blend, CS: Cabernet Sauvignon; SB: Sauvignon Blanc; WB, white blend.

In conclusion, to measure wine carbonyls, sulfur dioxide was successfully used to inhibit aldehyde formation by suppressing oxidation, greatly simplifying the operation by avoiding the need to exclude oxygen. It might be possible to run the treatment at higher temperatures or for a longer time by supplementing the SO_2 during the hydrolysis and derivatization step. In addition, hydrolysis of the bisulfite adducts was carried out using acid, replacing the standard alkaline hydrolysis. This removed one step as the hydrolysis and derivatization steps were carried out simultaneously, streamlining the procedure. The derivatives prepared in this manner were shown to be stable for a usable storage period, allowing HPLC analysis without concern for changes in observed levels. This approach offers many advantages over current practices including simpler operations, shorter reaction times, and suitable stability of the prepared samples at ambient temperature. With renewed interest in wine oxidation chemistry, this provides an efficient method for analysis of multiple key oxidation products, and it might be useful for the analysis of other foods containing SO_2.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.talanta.2014.11.046.

References

[1] M.A. Amerine, C.S. Ough, Methods for Analysis of Musts and Wines, John Wiley & Sons, New York, 1980.
[2] A.L. Waterhouse, S.E. Ebeler, Chemistry of Wine Flavor, Distributed by Oxford University Press, New York, 1998.
[3] L. Prokai, S. Szarka, X. Wang, K. Prokai-Tatrai, J. Chromatogr. A 1232 (2012) 281–287.
[4] L.C. de Azevedo, M.M. Reis, G.E. Pereira, G.O. da Rocha, L.A. Silva, J.B. de Andrade, J. Sep. Sci. 32 (2009) 3432–3440.
[5] M.P. Cruz, I.M. Valente, L.M. Gonçalves, J.A. Rodrigues, A.A. Barros, Anal. Bioanal. Chem. 403 (2012) 1031–1037.
[6] J.N. Jackowetz, R.M. de Orduna, Food Chem. 139 (2013) 100–104.
[7] R.J. Elias, V.F. Laurie, S.E. Ebeler, J.W. Wong, A.L. Waterhouse, Anal. Chim. Acta 626 (2008) 104–110.
[8] C. Flanzy, Enología: Fundamentos Científicos y Tecnológicos, Mundi-Prensa Libros, Madrid, Spain, 2000.
[9] V. Ferreira, P. Hernandez-Orte, A. Escudero, R. Lopez, J. Cacho, J. Chromatogr. A 864 (1999) 77–88.
[10] S.K. Frivik, S.E. Ebeler, Am. J. Enol. Vitic. 54 (2003) 31–38.
[11] A. Escudero, E. Asensio, J. Cacho, V. Ferreira, Food Chem. 77 (2002) 325–331.
[12] C. Saucier, D. Little, Y. Glories, Am. J. Enol. Vitic., 48, 1997370–373.
[13] C. Timberlake, P. Bridle, Am. J. Enol. Vitic. 27 (1976) 97–105.
[14] E. Gómez-Plaza, M. Cano-López, Food Chem. 125 (2011) 1131–1140.
[15] V. Atanasova, H. Fulcrand, V. Cheynier, M. Moutounet, Anal. Chim. Acta 458 (2002) 15–27.
[16] J. Bakker, C.F. Timberlake, J. Agric. Food Chem. 45 (1997) 35–43.
[17] H. Fulcrand, C. Benabdeljalil, J. Rigaud, V. Cheynier, M. Moutounet, Phytochemistry 47 (1998) 1401–1407.
[18] P. Ribéreau-Gayon, P. Pontallier, Y. Glories, J. Sci. Food Agric. 34 (1983) 505–516.
[19] T. Tanaka, R. Takahashi, I. Kouno, G.-i. Nonaka, J. Chem. Soc. Perkin Trans. 1 (1994) 3013–3022.
[20] M.A. Amerine, C.S. Ough, (1980).
[21] L. Burroughs, A. Sparks, J. Sci. Food Agric. 15 (1964) 176–185.
[22] E. Crowell, J. Guymon, J. AOAC Int. 46 (1963) 276–284.
[23] J.-C. Barbe, G. de Revel, A. Joyeux, A. Lonvaud-Funel, A. Bertrand, J. Agric. Food Chem. 48 (2000) 3413–3419.
[24] A.G.H. Lea, G.D. Ford, S. Fowler, Int. J. Food Sci. Technol. 35 (2000) 105–112.
[25] M. Vogel, A. Büldt, U. Karst, Fresenius J. Anal. Chem. 366 (2000) 781–791.
[26] S.D. Richardson, T.V. Caughran, T. Poiger, Y. Guo, F.G. Crumley, Ozone: Sci. Eng. 22 (2000) 653–675.
[27] E.J. Bartowsky, P.A. Henschke, Int. J. Food Microbiol. 96 (2004) 235–252.
[28] J.C. Nielsen, M. Richelieu, Appl. Environ. Microbiol. 65 (1999) 740–745.
[29] L.F. Burroughs, G.C. Whitting, The sulfur dioxide combining power of cider, Ann. Report Agr. Hort. Res. Station, Long Ashton, Bristol, England, 1960, p. 144.
[30] L.F. Burroughs, A.H. Sparks, J. Sci. Food Agric. 24 (1973) 187–198.
[31] R.C. Weast, Am. J. Med. Sci. 257 (1969) 423.
[32] H. Wildenradt, V. Singleton, Am. J. Enol. Vitic. 25 (1974) 119–126.
[33] A.L. Waterhouse, V.F. Laurie, Am. J. Enol. Vitic. 57 (2006) 306–313.
[34] M. Behforouz, J.L. Bolan, M.S. Flynt, J. Org. Chem. 50 (1985) 1186–1189.
[35] R. Boulton, V. Singleton, L. Bisson, R. Kunkee, Principles and Practices of Wihemaking, Champman & Hall, New York, 1995.
[36] J.C. Danilewicz, Am. J. Enol. Vitic. 58 (2007) 53–60.
[37] J.C. Danilewicz, Am. J. Enol. Vitic. 64 (2013) 316–324.
[38] R.J. Elias, A.L. Waterhouse, J. Agric. Food Chem. 58 (2010) 1699–1707.
[39] J.V. McArdle, M.R. Hoffmann, J. Phys. Chem. 87 (1983) 5425–5429.
[40] D.X. Ho, K.-H. Kim, Environ. Monit. Assess. 180 (2011) 163–176.