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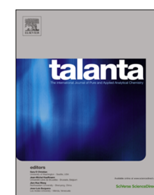

A rapid, one step preparation for measuring selected free plus SO_2-bound wine carbonyls by HPLC-DAD/MS

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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.

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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.

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