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Evaluation of the Formation and Stability of Hydroxyalkylsulfonic Acids in Wines

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The presence of carbonyl compounds (CCs) in wines has sparked the interest of researchers in several countries. The quantification of some of these compounds has been used as a parameter of quality for many fermented beverages. Although present in minute quantities (except for acetaldehyde), they have a strong olfactory impact. In addition, the CCs found in wines have a strong affinity for bisulfite and can form stable adducts, which will also interfere in the characteristics of aroma. The greatest challenge, however, is to predict which CCs have the strongest affinity for S(IV) and what conditions favor this interaction. To better understand the reaction of CC–bisulfite adduct formation (HASA), this study has evaluated the profile of 22 CCs in a "synthetic wine" containing bisulfite and in 10 real samples of different wines from the São Francisco Valley, northeastern Brazil. On the basis of principal component analysis (PCA) and dissociation constants, the results revealed that aliphatic aldehydes form adducts with S(IV), whereas ketones, cyclic aldehydes, and *trans*-alkenes interact weakly and are found predominantly in their free form. These results revealed also that pH 10 and 11 were defined as the most appropriate for CC–SO_2 adduct dissociation, and the total CCs were quantified reliably.

KEYWORDS: Hydroxyalkylsulfonic acids; carbonyl compounds; bisulfite; wine; São Francisco Valley, Brazil

INTRODUCTION

Sulfur-based compounds [$Na_2S_2O_5$ or $K_2S_2O_5$ (sodium or potassium metabisulfite) and/or $KHSO_3$ (potassium bisulfite)] have been used by wineries to solve problems related with oxidation and microbial interactions in wine (*1*–*3*). In aqueous solutions, these compounds produce various species of S(IV) involved in the equilibrium depending mainly on the pH.

These S(IV) species compete with molecular oxygen for the chemical groups susceptible to oxidation and then inhibit some reactions caused by molecular oxygen. As the use of these compounds became widespread, so did the concern about their damaging effect on health, particularly because they are associated with problems of asthma in people who consume alcoholic beverages (*4*, *5*). In terms of the SO_2 concentration, the levels of S(IV) compounds allowed by the EU (European Union) and the Organización Internacional de la Viña e del Vino–International Grape and Wine Organization (OIV) for some types of wine vary from 160 to 260 mg L^{-1} and from 175 to 360 mg L^{-1}, respectively (*6*). However, according to the literature, concentrations of <10 mg L^{-1} suffice to trigger asthma attacks (*7*).

There is the establishment of a chemical equilibrium between "free SO_2" and "bound SO_2" forms in sulfited wines (*6*). The concentrations of free SO_2 found in different types of wine may vary as a function of the composition of the beverage, the quantity of bisulfite and/or metabisulfite added, and the greater or lesser facility for interaction between S(IV) and some components of the matrix. Several carbonyl substances are present among these components, such as formaldehyde, acetaldehyde, glyoxal, methylglyoxal, pyruvic acid, xylose, α-ketoglutaric acid, galacturonic acid, glucuronic acid, 2-ketogluconic acid, 2,5-diketogluconic acid, 5-ketofructose, and hydroxypropanedial (*6*, *8*, *9*). Because musts and wines contain significant levels of these carbonyl compounds (CCs), this combination should be considered, because it might influence the reduction of the concentration

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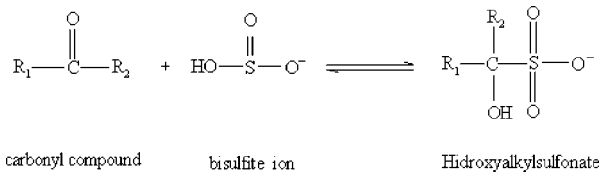
of free SO_2 (*6*). Another aspect to be taken into account is the fact that the adduct formed between these compounds and S(IV) is usually not very stable, except for the interaction with acetaldehyde. The equilibrium of the adduct's formation depends on the temperature, the pH of the medium, and the type of chemical constituents that combine with S(IV). Moreover, any change in the composition of the wine inevitably affects this equilibrium, which is demonstrated in eq 1 (*3, 6*):

$$SO_2 + H_2O \underset{}{\overset{pk = 1.81}{\rightleftharpoons}} \underset{\text{(predominant form in wine)}}{HSO_3^- + H^+} \overset{pk = 6.91}{\rightleftharpoons} SO_3^{2-} \quad (1)$$

In a typical wine possessing pH from 3 to 4, the predominant form among the S(IV) species is that of the bisulfite ion (*6, 8, 10*). **Table 1** shows the relative abundance of S(IV) species such as free SO_2 [active S(IV) species] and HSO_3^-, over pH from 3 to 4. The data make evident that for higher pH values the amount of free SO_2 decreases, justifying a larger sulfiting when must or wine is at weak acidic conditions. The different forms that sulfur dioxide can assume in wine are shown in **Figure 1**. Briefly, in the region ranging from (a) to (b), on the left, is present the free SO_2 form, which may be transformed into HSO_3^- as a function of the pH. The line at (b) divides the free SO_2 form region from the SO_2 bound to carbonyl compounds from wine ranges, excluding acetaldehyde. This line may be moved in either direction by changes of temperature and SO_2 level available in wine. On the right, the range (c–d) represents the SO_2 bound to acetaldehyde. This is a fixed line because it depends only on the acetaldehyde level of the wine (*6*).

In aqueous solutions, S(IV) compounds can bind to carbonyl compounds in various ways, and one of these combinations may lead to the formation of addition compounds known as hydroxyalkylsulfonic acids (HASA) (eq 2) (*11, 12*).



(2)

The affinity of a carbonyl compound for bisulfite is represented by the dissociation constant of the corresponding addition compound defined according to the law of mass action. The apparent equilibrium constant (K_d) is given by

$$K_d = \frac{[S][X - x]}{[x]} \quad (3)$$

where [S] = concentration of free S(IV) species in any form, [X] = total concentration of carbonyl compound (free + bound), and [x] = concentration of undissociated carbonyl bisulfite.

Burroughs and co-workers (*13–16*) studied intensively the sulfite-binding power focused on the equilibrium constants for dissociation of some carbonyl bisulfite compounds present in apple juice, ciders, and wines. The carbonyl compounds evaluated by these authors were acetaldehyde, pyruvic acid, 2-ketoglutaric acid, L-xylosone, D-*threo*-2,5-hexodiulose, 2,5-diketogluconic acid, 2-ketogluconic acid, and galacturonic acid. The apparent equilibrium constants remain almost unchanged over pH 3 and 4 for each CC, ranging from 1.5×10^{-6} to 1.6×10^{-2}. Acetaldehyde was the most stable carbonyl bisulfite compound evaluated [K_d (pH 3) = 1.5×10^{-6} and K_d (pH 4) = 1.4×10^{-6}]; its dissociation constant value was too small to allow acetaldehyde in the free form in solution (*6, 17, 18*). According to Burroughs (*13*), at a free SO_2 concentration of 6.4 mg L^{-1}, 98% of acetaldehyde in solution would be in the bound form, whereas galacturonic acid was the least stable [K_d (pH 3) = 1.6×10^{-2} and K_d (pH 4) = 2.1×10^{-2}].

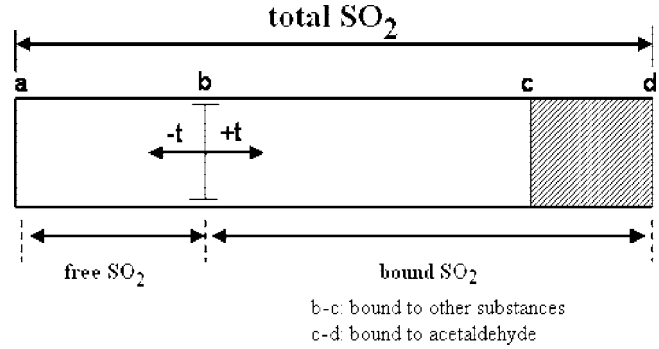


Figure 1. Representation of different possibilities of combination of SO_2 in the wine (adapted from Ribereau-Gayon, 2003).

Table 1. Relative Abundance of Two Free S(IV) Species as a Function of pH at 20 °C in Aqueous Solution (*6*)

pH	free SO_2 (%)	HSO_3^- (%)
3.00	6.06	94.94
3.10	4.88	95.12
3.20	3.91	96.09
3.30	3.13	96.87
3.40	2.51	97.49
3.50	2.00	98.00
3.60	1.60	98.40
3.70	1.27	98.73
3.80	1.01	98.99
3.90	0.81	99.19
4.00	0.64	99.36

Acetaldehyde, in particular, reacts rapidly with sulfite or bisulfite ions, forming an addition product that is not volatile and, thus, reducing its olfactory perception (*4*). This justifies the use of S(IV) compounds to disguise the excess of this aldehyde in wine. The adduct thus formed, α-hydroxyethanesulfonic acid, consists of a strong acid species involved in the equilibrium. On the basis of the typical acid strengths of other sulfonic acids, the first acid dissociation constant of α-hydroxyethanesulfonic acid is expected to be >0.1 and the second acid dissociation constant has also been determined as 5.0×10^{-11} (*19*).

Carbonyl compounds of higher molar masses can also form HASA. The stability of these acids also depends on the structure of the bound CC, the pH, and the temperature of the medium (*18*). It is essential to know the concentrations of the acids formed with more complex carbonyl compounds in order to understand the equilibrium of the adduct formation reactions and to allow the quantification of total CCs present in samples of wine or of other fermented beverages.

Free aldehydes can be determined directly by high-performance liquid chromatography (HPLC), after derivatization reaction with 2,4-dinitrophenylhydrazine (2,4-DNPH) solution to form the respective 2,4-dinitrophenylhydrazones, and then quantifying them by comparison to standard solutions containing known concentrations of the CCs under study (*20–25*). Derivatization is necessary due to the relative instability of the carbonyl chemical function in view of the complex medium of wine (*20–26*). The formation of hydrazones must occur in acidic conditions, because the acidic catalysis activates the carbon of

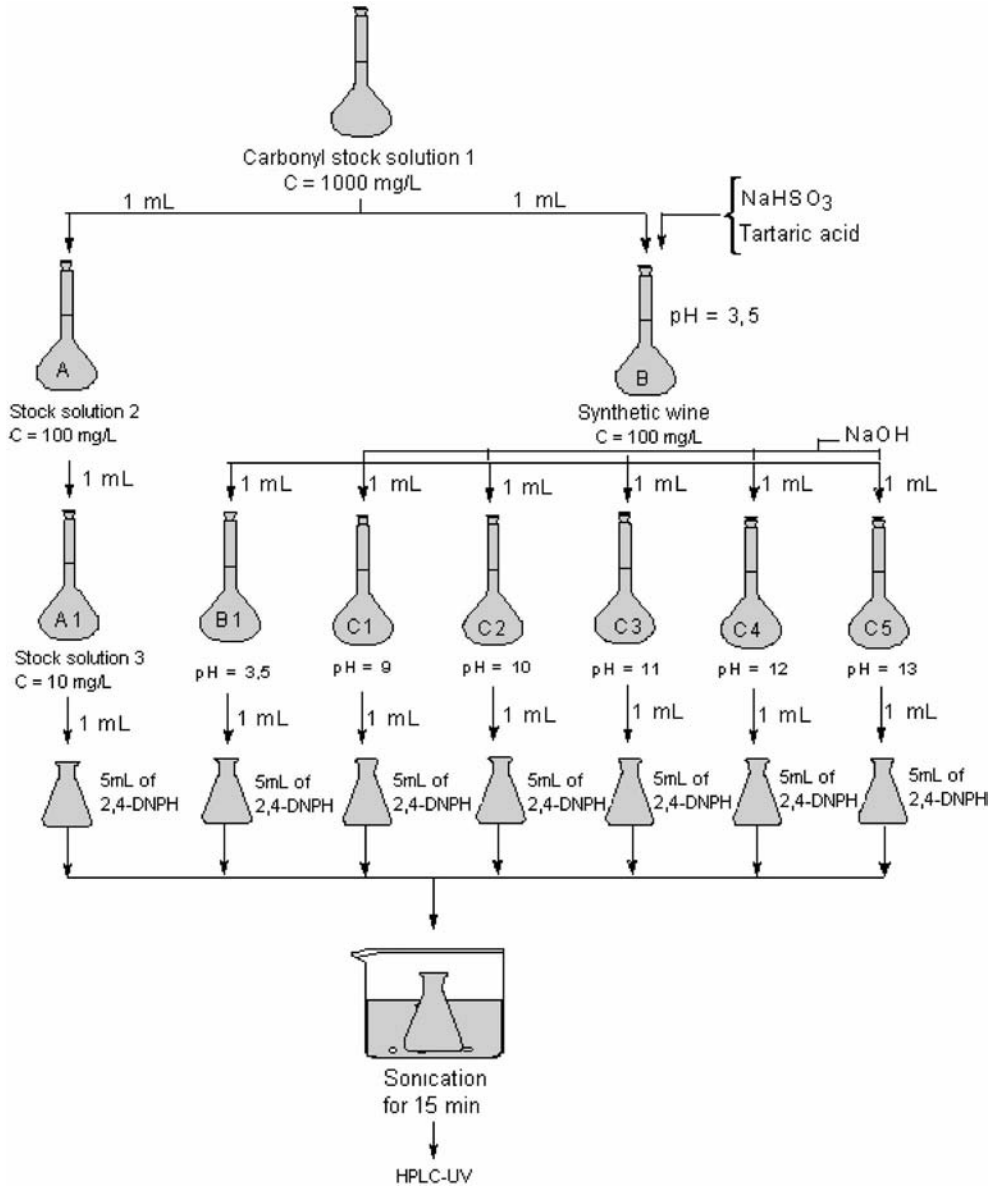


Figure 2. Diagram of the preparation of a sample to obtain the HASA adduct in "synthetic wine".

the carbonyl group by protonation of oxygen to yield a stronger electrophile that can be attacked by a weak nucleophile, such as 2,4-DNPH. According to Veloso and co-workers (*24*, *25*, *27*), the pH influence is due to a competition between nucleophilicity and basicity of 2,4-DNPH and the electrophilic nature of the carbon of the carbonyl group. For too low pH values, 2,4-DNPH acts as a base and it is protonated, decreasing its nucleophilic action. For higher pH values, the reactivity of the carbonyl group decreases. In this regard, it is necessary to establish the optimum pH to equilibrate both factors. Veloso and co-workers found the ideal range of pH to yield the best carbonyl compound recovery to lie in the interval of 1.5–2.2.

A method to determine formaldehyde, acetaldehyde, hydroxymethanesulfonic acid (AHMS), and α-hydroxyethanesulfonic acid (AHES) in white wine samples based on 2,4-DNPH derivatives was developed by de Oliveira and de Andrade (*21*). Total formaldehyde and acetaldehyde were measured by dissociating the AHMS and AHES into formaldehyde, acetaldehyde, and bisulfite with a strong base and reacting the aldehydes with 2,4-DNPH. Free formaldehyde and acetaldehyde are similarly determined in another aliquot, but without addition of the alkaline solution. The difference between the total and

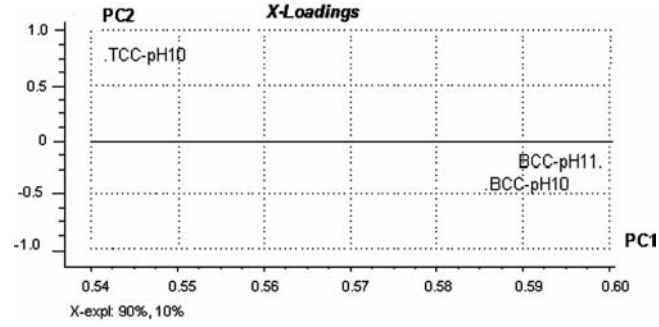


Figure 3. Loadings diagram of the first two principal components (PC1 and PC2), indicating the variables responsible for the grouping of the samples into subgrouups (FCC, free; BCC, bound; TCC, total).

free aldehydes gives the bound aldehydes or the respective AHMS and AHES contents in the wine samples.

In the quantification of total CCs based on the decomposition of the HASA adduct, the literature (*21*) suggests that the sample first be acidified with HCl and slightly heated (50 °C) to eliminate excess SO_2. NaOH solution should then be added to the sample to ensure the medium is sufficiently alkaline to

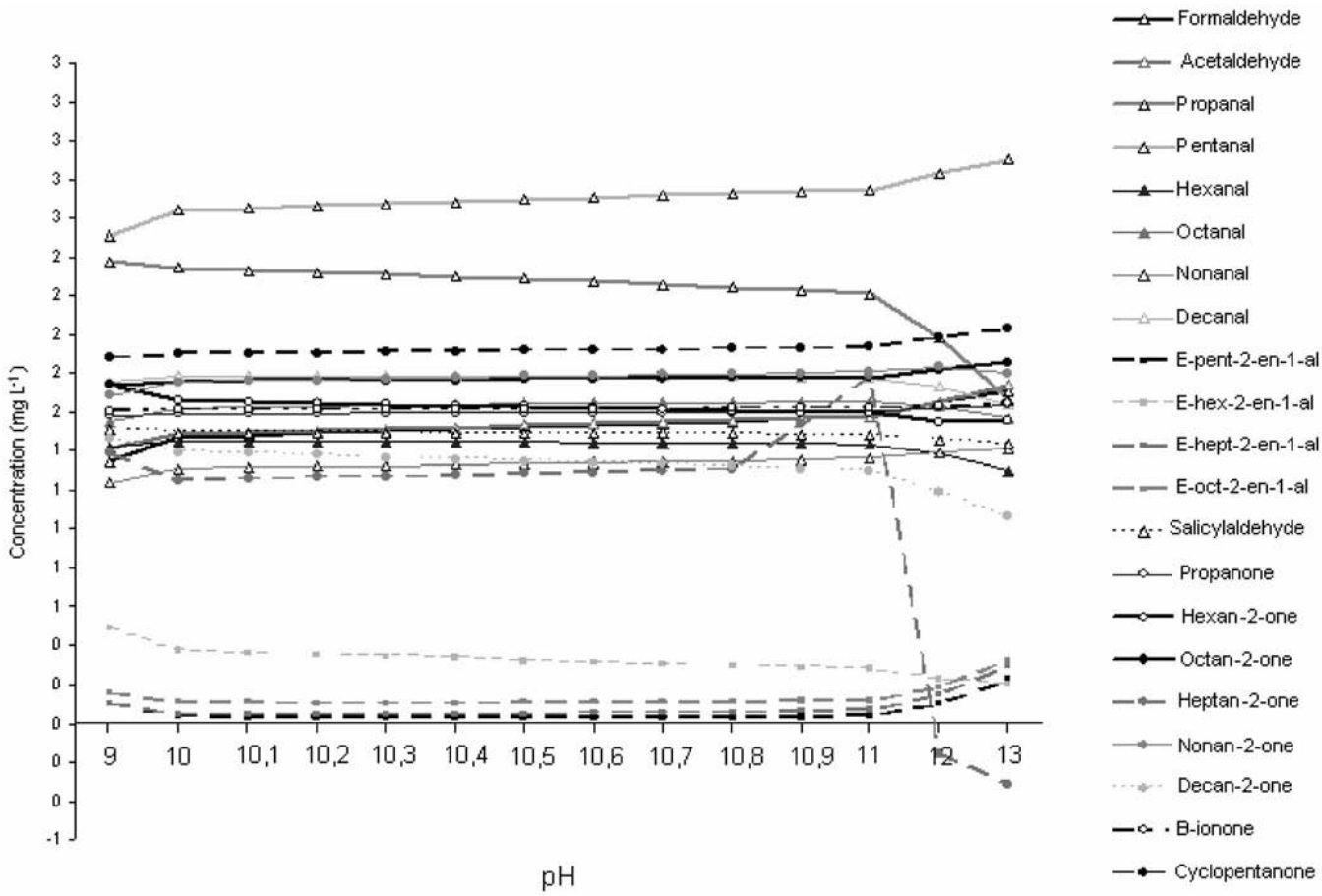


Figure 4. Variation of the CC concentration as a function of pH in synthetic wine, using the quadratic equations obtained by regression.

decompose the HASA. This stage is followed by the derivatization and quantification procedure, as described for free CCs. The HASA concentrations are determined by the difference between contents of total and free CCs, for each CC (*21, 28*).

Although some studies on the formation of HASA from main high molar mass CCs found in wines, apple juice, and cider are reported in the literature (*8, 14–16*), a better understanding of CCs and SO_2 interactions as well as the ideal pH for the HASA dissociation is still needed. Therefore, the main focus of this study was to evaluate the influence of the molecular structure of aldehydes and ketones on the formation of HASA and to define the best range of alkaline pH within which it is possible to obtain the highest analytical signal for the determination of total CCs. To prevent interferences from the matrix, the tests were carried out on "synthetic wine" and latter applied to real samples of both white and red wines.

MATERIALS AND METHODS

Preparation of Standards and Samples. The reagents and standards utilized were of analytical grade and the solvents of HPLC grade. The solutions were prepared using deionized water obtained with the Millipore system.

The profile of different aldehydes and ketones in the presence of bisulfite was investigated in a solution that reproduced some of the conditions found in wine, such as the alcohol content, bisulfite content, and pH. This solution, which was dubbed "synthetic wine", was prepared by mixing a solution of CC standards (C_{final} = 10 mg L^{-1}), sodium bisulfite (C_{final} = 50 mg L^{-1}), and tartaric acid, which was added to adjust the pH close to that of wine, that is, 3.5, and completed with 11% ethanol (11:89, ethanol/deionized water, v/v).

The 22 carbonyl compounds studied here were formaldehyde, acetaldehyde, propanal, pentanal, hexanal, octanal, nonanal, decanal, *E*-pent-2-en-1-al, *E*-hex-2-en-1-al, *E*-hept-2-en-1-al, *E*-oct-2-en-1-al, benzaldehyde, salicylaldehyde, propanone, hexan-2-one, heptan-2-one, octan-2-one, nonan-2-one, decan-2-one, β-ionone, and cyclopentanone. All of these compounds have been cited as constituents of the volatile fraction of wines (*8, 9, 26, 29–32*).

Standard solutions of each CC were prepared to obtain a concentration of 1000 mg L^{-1}. An aliquot of 1 mL of this solution was transferred to a volumetric flask A and another to flask B with the same capacity (**Figure 2**). In both cases, the volumes were completed with 11% ethanol solution to give a final concentration 100 mg L^{-1} for each CC. Tartaric acid and sodium bisulfite (to favor the formation of the AHAS adduct) were added to flask B, and it was dubbed "synthetic wine" (SW).

One milliliter of solutions A and B was transferred to flasks A1 and B1 and completed with 11% ethanol solution to give a final concentration of 10 mg L^{-1}. Then, 1 mL from each flask was taken and transferred to amber-colored bottles containing 5 mL of 2,4-DNPH at 0.4%, acidified with phosphoric acid, to enable the CC to be derivatized. The mixtures were then sonicated for 15 min and injected into the HPLC system. The free carbonyl compounds (FCC) contained in the solutions of flasks A1 and B1 were then analyzed. The solution of flask A1 was considered the bisulfite blank (BB) and was used as reference in the calculation of the concentrations.

Equal volumes (1 mL) of synthetic wine (B) were then transferred to volumetric flasks C1, C2, C3, C4, and C5. The pH of the solutions contained in the flasks was adjusted to 9, 10, 11, 12, and 13, respectively, by the addition of 1 mol L^{-1} of NaOH. All flasks then were completed with 11% ethanol solution. An aliquot of 1 mL of the solution contained in each flask was derivatized with 5 mL of 2,4-DNPH 0.4% solution and used for the analysis of the total carbonyl compounds (TCC).

To evaluate the role of both an individual CC by itself and all CCs together with respect to adduct formation at different pH values, the above-described sample procedure was initially prepared with each of

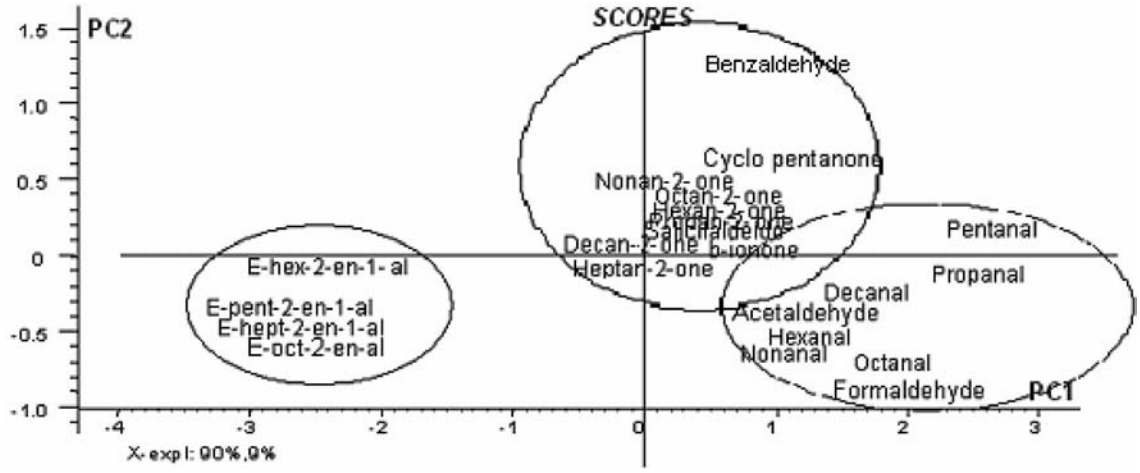


Figure 5. Scores diagram of the first two principal components (PC1 and PC2), indicating the grouping of the samples into three subgroups: aldehydes, ketones, and *trans*-alkenes.

Table 2. Contribution of the Autoscores of Variables to PC1 and PC2

variable	PC1	PC2
BCC-pH11	0.599	−0.298
BCC-pH10	0.588	−0.467
BCC-pH10	0.544	0.832

Table 3. Accumulative Percentage Variance into PC1 and PC2

	PC1		PC2	
variable	calibration	validation	calibration	Validation
PCA	90.00	83.30	99.60	98.46
BCC-pH11	96.78	94.62	99.34	97.50
BCC-pH10	93.20	88.65	99.48	97.98
TCC-pH10	80.01	66.05	99.98	99.95

the CCs individually and then analyzed. Next the same sample procedure was repeated for the preparation of a solution containing all of the CCs jointly, and it was also subjected to analysis. The latter was prepared with all 22 CCs at a concentration of 1000 mg L^{-1}, followed by the dilution steps as already described for each individual CC solution.

Free S(IV) species determinations were made by titrating the equilibrated solutions containing carbonyl bisulfite compounds with iodine standard solution. The start solutions were prepared following the same procedure already described, with 100 mg L^{-1} of each CC and 500 mg L^{-1} HSO_3^-, keeping the same ratio (CC:HSO_3^-) of all solutions used here. The pH was adjusted at 3.5 with tartaric acid.

For real sample analysis five bottles of white wine and five bottles of red wine were acquired from wineries in the São Francisco Valley, northeastern Brazil. Free and total CCs were determined in each bottle, and all analyses were performed in triplicate, using the same procedure described above. TCC determination was carried out at pH 11.

HPLC Analysis. The carbonyl compounds and their respective hydroxyalkylsulfonic acids were analyzed by high-performance liquid chromatography (HPLC) with a reversed-phase gradient system composed of a Perkin-Elmer series 200 pump, a Rheodyne injector valve with a 20 μL loop, a Perkin-Elmer series 200 UV–vis (model 5100) detector equipped with a deuterium lamp ($\lambda = 365$ nm), and an Intralab (model 4290) integrator. The compounds were separated in a LiChroCART RP-18 (250 × 0.4 mm; 5 μm) column by passage of the mobile phases A (74.5% methanol/0.5% acetonitrile/25% water, v/v/v) and B (100% methanol), according to the following schedule: 12 min, 100% A; 12 min, 100% A→B; 3 min, 100% B; 10 min, 100% B→A.

Statistical Treatment. CC quantification was made by applying the external standardizing method, through standard analytical curves, considering the integrated area of chromatographic peaks.

The statistical treatment data of the "synthetic wine" was based on a multivariate analysis of the data matrix. The original data matrix consisted of 22 samples and 10 variables in different pH values (the 22 × 10 format). The samples correspond to the carbonyl compounds evaluated, whereas the concentrations of both bound (BCC) and total carbonyl compounds (TCC) represent the variables. The concentrations of BCCs and TCCs were measured in synthetic wine at pH 9, 10, 11, 12, and 13. FCC values were not used in the statistical analysis.

The method employed was principal component analysis (PCA), using the computational program Unscrambler, version 7.6. In this method, the data are explored and compacted, grouping similar samples and selecting the most relevant variables. First, a preprocessing of autoscaling was performed in order to obtain the same loading for all variables. In this way, a symmetric and quadratic correlation matrix with diagonal elements with values equal to 1 was reached, confirming high correlations among them. Second, by performing the PCA, these variables are grouped to a new variable (the principal component), directed to the axis of the greatest spreading of data and then decreasing the variables dimension, which consequently provokes the reduction of the number of variables. The diagrams of scores and loadings were analyzed.

In the multivariate analysis the reduction processing of spatial dimension provokes a consequent decrease of the number of variables. The goal is to get parameters that are not highly correlated to each other and then samples are grouped and distinguished themselves. The grouping was performed as follow: (a) decomposition of singular values (DSV); (b) diagonalizing of correlation matrix. First, the original data matrix (X) was decomposed into three matrices (**A**, **B**, and **C**), DSV Method, according to eq 4; matrixes **A** and **C** are squared and orthonormals, that is, the rows **A** and **C** are orthogonal to each other and normalized.

$$X = \mathrm{A} \cdot \mathrm{B} \cdot \mathrm{C}^{\mathrm{T}} \tag{4}$$

The B matrix is diagonal and rectangular, being of singular values in the diagonal to all elements out of the zero diagonal. In this way, the multiplication factor **A** · **B** represents the scores matrix **T** and **C** corresponds to the loadings matrix. The rows from **C** matrix are autovectors of matrix $\mathbf{X^T} \cdot \mathbf{X}$, and the rows from matrix **A** are autovectors of matrix $\mathbf{X} \cdot \mathbf{X^T}$. Then the diagonalization of matrix correlation was made, where the autovectors **C** and **K** were calculated by using eq 5. The autoscores **K** are the diagonal and their elements are the respective squared singular values (λ_k) according to eq 6.

$$\mathrm{X}^{\mathrm{T}} \cdot \mathrm{X} \cdot \mathrm{C} = k \cdot \mathrm{C} \tag{5}$$

$$\lambda_k = (S_{kk})^2 \tag{6}$$

Each element λ_k from the diagonal refers to variance of original data, which is described by the kth principal component. The information

Table 4. Concentrations (Milligrams per Liter) of Free, Bound, and Total CCs in "Synthetic Wine", Analyzed Individually[a]

CC	BB	FCC	TCC-pH9	TCC-pH10	TCC-pH11	TCC-pH12	TCC-pH13	BCC-pH9	BCC-pH10	BCC-pH11	BCC-pH12	BCC-pH13
formaldehyde	1.67	0.21	1.22	1.51	1.56	1.83	1.56	1.01	1.30	1.35	1.62	1.34
acetaldehyde	1.67	0.96	1.30	1.54	1.78	1.44	1.79	0.34	0.58	0.82	0.48	0.83
propanal	1.67	0.57	1.54	1.51	1.66	1.12	1.09	0.96	0.93	1.08	0.54	0.76
pentanal	1.67	0.78	1.46	1.46	1.67	1.62	1.67	0.68	0.68	1.04	0.84	1.53
hexanal	1.67	0.56	1.47	1.48	1.36	1.26	1.39	0.91	0.92	0.80	0.70	0.83
octanal	1.67	0.34	1.46	1.65	1.80	1.55	1.56	1.12	1.31	1.46	1.21	1.22
nonanal	1.67	0.52	1.20	1.33	1.33	1.47	1.36	0.68	0.81	0.81	0.95	0.84
decanal	1.67	0.75	1.85	1.80	1.59	1.72	1.69	1.10	1.04	0.83	0.97	0.94
E-pent-2-en-1-al	1.67	1.65	0.04	0.06	0.08	0.12	0.19	nd	nd	nd	nd	nd
E-hex-2-en-1-al	1.67	1.78	0.54	0.38	0.24	0.20	0.24	nd	nd	nd	nd	nd
E-hept-2-en-1-al	1.67	1.59	0.04	0.06	0.12	0.18	0.26	nd	nd	nd	nd	nd
E-oct-2-en-1-al	1.67	1.53	0.13	0.09	0.16	0.22	0.29	nd	nd	nd	nd	nd
benzaldehyde	1.67	1.66	1.55	1.39	1.67	1.59	1.61	nd	nd	nd	nd	nd
salicylaldehyde	1.67	1.50	1.43	1.58	1.50	1.46	1.41	nd	0.08	nd	nd	nd
propanone	1.67	1.59	1.60	1.59	1.59	1.60	1.66	0.01	nd	nd	0.01	0.07
hexan-2-one	1.67	1.57	1.70	1.70	1.57	1.50	1.54	0.12	0.12	nd	nd	nd
heptan-2-one	1.67	1.30	1.24	1.11	1.23	1.48	1.44	nd	nd	nd	0.19	0.14
octan-2-one	1.67	1.83	1.67	1.77	1.89	1.77	1.84	nd	nd	0.06	nd	0.01
nonan-2-one	1.67	1.92	1.67	1.67	1.79	1.73	1.71	nd	nd	nd	nd	nd
decan-2-one	1.67	1.49	1.56	1.35	1.46	0.73	1.32	0.07	nd	nd	nd	nd
β-ionone	1.67	1.58	1.63	1.60	1.60	1.60	1.67	0.04	0.02	0.02	0.02	0.08
cyclopentanone	1.67	1.39	1.10	1.41	1.67	1.36	1.35	nd	nd	0.28	nd	nd

[a] nd, not detected; BB, bisulfite blank; FCC, free; TCC, total; BCC, bound.

Table 5. Concentrations (Milligrams per Liter) of Free, Bound, and Total CCs in "Synthetic Wine", Analyzed Jointly[a]

CC	BB	FCC	TCC-pH9	TCC-pH10	TCC-pH11	TCC-pH12	TCC-pH13	BCC-pH9	BCC-pH10	BCC-pH11	BCC-pH12	BCC-pH13
formaldehyde	1.67	1.09	1.44	1.79	1.91	1.55	1.53	0.35	0.70	0.82	0.46	0.44
acetaldehyde	1.67	0.81	1.20	1.53	1.67	1.56	1.72	0.40	0.74	0.88	0.76	1.32
propanal	1.67	0.70	1.45	1.74	1.72	1.74	1.72	0.75	1.04	1.02	1.04	1.02
pentanal	1.67	0.43	0.31	1.48	1.67	1.65	1.64	nd	1.04	1.23	1.22	1.52
hexanal	1.67	0.68	1.51	1.80	1.88	1.50	1.46	0.83	1.11	1.19	0.81	0.77
octanal	1.67	0.81	1.70	1.91	1.98	1.70	1.70	0.89	1.10	1.17	0.89	0.89
nonanal	1.67	0.92	1.16	1.62	1.67	1.49	1.49	0.26	0.71	0.74	0.58	1.76
decanal	1.67	1.33	1.00	1.55	1.67	1.33	1.33	nd	0.22	0.33	nd	nd
E-pent-2-en-1-al	1.67	1.53	0.17	0.24	0.12	0.12	0.24	nd	nd	nd	nd	nd
E-hex-2-en-1-al	1.67	1.42	0.23	0.34	0.15	0.15	0.34	nd	nd	nd	nd	nd
benzaldehyde	1.67	1.32	1.37	1.80	1.80	1.72	1.59	0.05	0.48	0.48	0.40	0.28
salicylaldehyde	1.67	1.42	1.40	1.43	1.43	1.40	1.50	nd	0.02	0.02	nd	0.08
propanone	1.67	1.55	1.59	1.54	1.61	1.61	1.67	0.04	nd	0.06	0.06	0.11
hexan-2-one + *E*-hept-2-en-1-al	1.67	1.47	1.55	1.50	1.55	1.55	1.61	0.08	0.03	0.08	0.08	0.14
heptan-2-one + *E*-oct-2-en-1-al	1.67	1.67	1.52	1.59	1.49	1.49	1.49	nd	nd	nd	nd	nd
octan-2-one	1.67	1.25	1.25	1.19	1.37	1.31	1.31	nd	nd	0.12	0.06	0.06
nonan-2-one	1.67	1.09	1.14	1.14	1.25	1.25	1.15	0.05	0.05	0.16	0.16	0.05
decan-2-one	1.67	0.96	1.03	0.90	1.03	1.03	0.90	0.06	nd	0.06	0.06	nd
β-ionone	1.67	0.81	0.86	0.81	0.97	0.86	0.86	0.05	nd	0.16	0.05	0.05
cyclopentanone	1.67	1.16	0.89	1.39	1.67	1.48	1.45	nd	0.23	0.92	0.34	0.43

[a] nd, not detected; BB, bisulfite blank; FCC, free; TCC, total; BCC, bound.

content in each principal component can be described by variance percentage (%Var_k). The total variance of the group is determined by summation of variance of each principal component. Thus, the first principal component always will contain the greatest percentage of information, as already described. It is necessary to consider all principal components that describe about 95% of the original information. In this moment, there are the significant principal components, yet their accumulated percentage variance (%$Var_{accumulated}$) can be calculate. After the PCA treatment, a cross-validation was executed to obtain the validated percentage of the analysis. The software Unscrambler, version 7.6, does the PCA using DSV and diagonalizing of correlation matrix, reaching a resulting modeling of calibration. This software also performs the internal cross-validation method by taking samples one by one with the goal of testing the prediction capacity of the proposed method. The cross-validation method was randomly based.

Using the same data matrix, a regression analysis was also carried out, selecting the mathematical model by the highest adjusted R^2 value and setting the significance of the regression coefficient at 5% by the *t* test. For the majority of studied CCs, the quadratic model presented the best fit. The regression analysis was carried out by the SAEG-UFV, version 8.1, program (*33*), and its main objective was to predict the role of concentration values of the studied CCs in synthetic wine as a function of pH values changing in alkaline range during TCC analysis and, in this way, to select the best pH (or pH range) for a satisfactory response in the study. Through observation of critical points in the obtained curves, we have defined the ideal range of pH to be applied in the preparation of samples.

RESULTS AND DISCUSSION

The determination of free carbonyl compounds relies on the assumption that carbonyl bisulfite compounds are stable in acidic medium; thus, bound CCs in the HASA adducts do not react with 2,4-DNPH to form hydrazone derivatives (*21*, *28*, *36*). Ang and co-workers (*36*) studied hydroxymethanesulfonic acid (HMSA) dissociation and 2,4-DNPH reaction at pH 2; because no HMSA dissociation was observed by them under this pH condition, no HCHO–2,4-DNPH derivatives were thus formed (*36*). On the other hand, the addition of a strong base to HMSA solution (pH 13) dissociates the adduct into free formaldehyde and bisulfite, allowing the derivatization reaction. In a previous

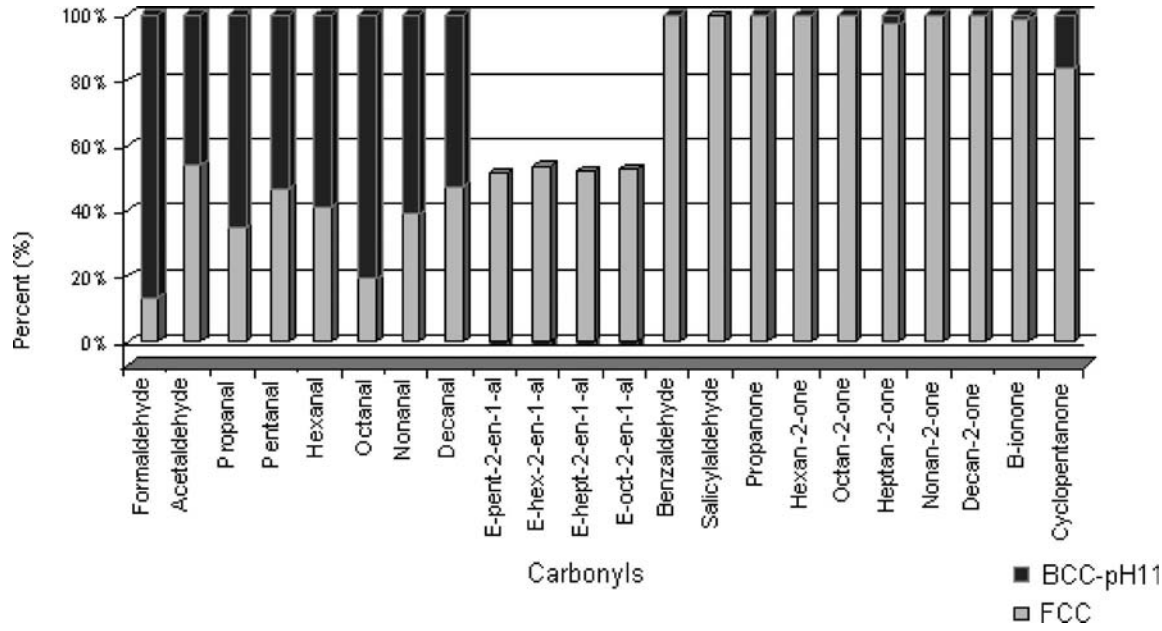


Figure 6. Percentage of FCC and BCC-pH11 in samples of "synthetic wine", analyzed individually (BCC, bound; FCC, free).

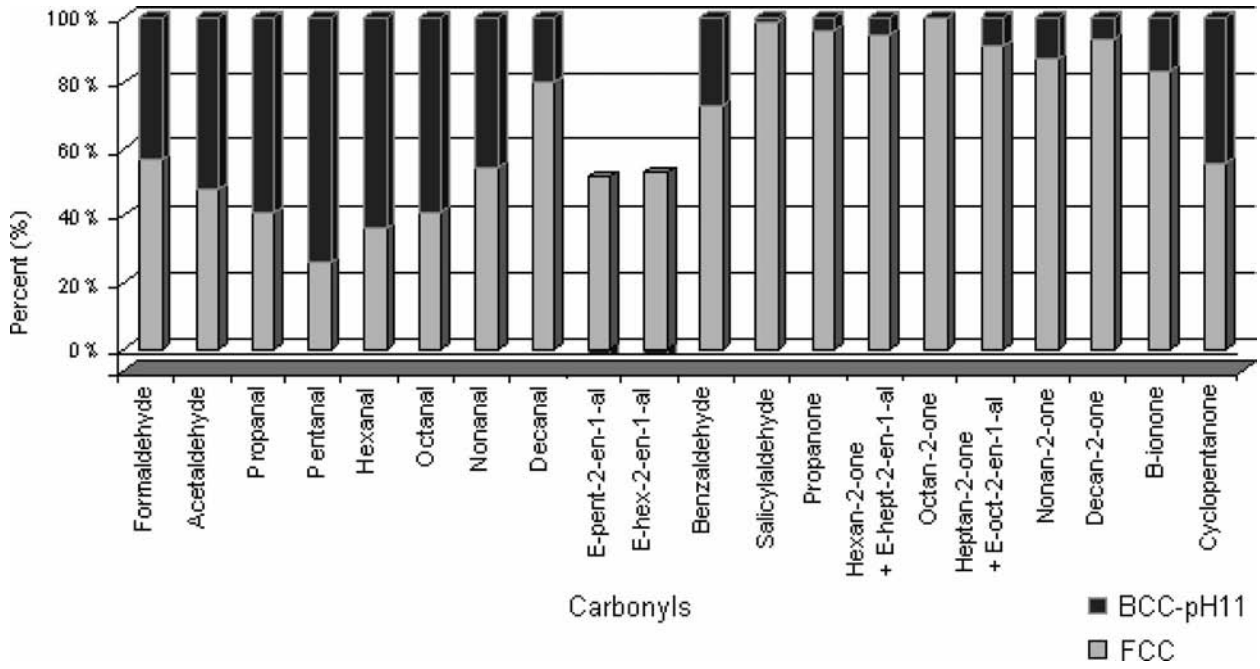


Figure 7. Percentage of FCC and BCC-pH11 in samples of "synthetic wine", analyzed jointly (BCC, bound; FCC, free).

work (*28*), we observed similar results for HMSA. This was also verified for acetaldehyde in which standard α-hydroxyethanesulfonic acid (HESA) solutions were added to 2,4-DNPH reagent in pH ranging from 2.53 to 5.50 and no H_3CCHO–2,4-DNPH derivatives were formed. Indeed, an alkaline pH is necessary to ensure the complete dissociation of the HASA adduct (the BCC form), thus allowing for the reliable quantification of the TCC present in the sample. Generally speaking, pH 10 and 11 were considered to be the most significant to obtain the best response in terms of TCC concentration. This pH range was defined after PCA and regression results. The loadings diagram (**Figure 4**) indicates that the variables TCC-pH10, BCC-pH10, and BCC-pH11 were selected, justifying the choice of pH 10 and 11 as the most important. This analysis showed that the other pH values, because they present autoscores smaller than those of pH values 10 and 11, were not selected; therefore, they do not present a significant relevance in the determination of concentrations of the studied CCs and their discrimination. In the same way, in **Figure 4**, which was obtained by regression, it is clear that these pH values are considered to be critical points of the curves because before pH 10 and after pH 11 there are changes in the curve trending (response either increases or decreases). This is not desired during a simultaneous analysis of all CCs in a real sample of wine. On the other hand, by using quadratic modeling to estimate the role of the response in the pH range 10–11, it could be noted that there were not significant differences among results. Therefore, this pH range could guarantee a complete dissociation of the adduct and ensure reliable results in the TCC analysis to determine CCs quantitatively.

The PCA revealed that the first two components were able to explain 99.6% of the total variance of the data set. The first principal component (PC1) explained 89.99% of the variance, whereas the second (PC2) explained 9.61%. After PCA, three

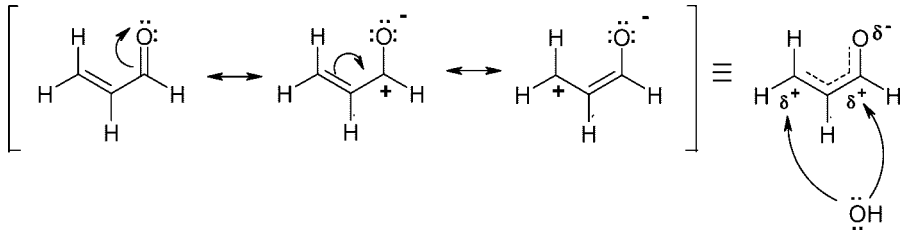


Figure 8. Resonance structures and resonance hybrid of α- and β-unsaturated aldehydes favorable to simple addition reaction (adapted from ref *28*).

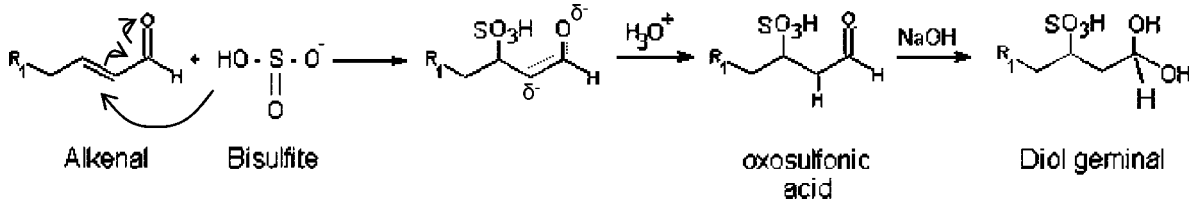


Figure 9. Conjugate addition, under Michael addition route, between an anion and an α,β-unsaturated carbonyl compound.

Table 6. Dissociation Constants (K_d) of Carbonyl Bisulfite Compounds at pH 3.5

CC	K_d
formaldehyde	1.09×10^{-7}
acetaldehyde	2.06×10^{-6}
propanal	7.70×10^{-6}
pentanal	8.00×10^{-6}
hexanal	3.45×10^{-6}
octanal	2.52×10^{-6}
nonanal	4.12×10^{-6}
decanal	4.46×10^{-6}
E-pent-2-en-1-al	8.25×10^{-4}
E-hept-2-en-1-al	1.57×10^{-4}
E-oct-2-en-1-al	8.48×10^{-4}
benzaldehyde	2.83×10^{-3}
hexan-2-one	1.13×10^{-4}
heptan-2-one	4.30×10^{-4}
decan-2-one	8.30×10^{-5}
β-ionone	2.07×10^{-4}

parameters were selected, and neither outlier was detected. The scores diagram (**Figure 5**) indicates that the samples were grouped into three categories: aldehydes, ketones, and alkenals. In PCA, scores situated close to each other present correlations that are statistically significant (*34*). The scores diagram revealed not only that the samples were separated into three groups but also that the unsaturated derivatives (alkenals) were separated from the other carbonyl compounds. The BCC-pH10 and BCC-pH11 variables (loadings diagram) contribute positively to PC1, which justifies that in the pH 10–11 the alkenals group (scores diagram) is distinguished from the other CCs group (the aldehydes and ketones groups) because the unsaturated aldehydes are located on the left quadrant with the least contribution to PC1. Also, this grouping is lower on the PC1 axis, characterized by a small negative contribution to PC2. Aldehyde and ketone samples tend to be presented mainly in the bound form in the pH range when compared to alkenals. Analyzing the data matrix, one can see that at pH 11, these CCs are practically in free form, which is congruent with the statistical analysis.

A joint analysis of the scores and loadings diagrams shows an indication for the interpretation of the experimental data. It was found that the aldehyde compounds group together in the lower quadrant (scores), so the variable BCC-pH11 (loadings) is responsible for explaining the aldehydes' profile. Therefore, the form bound at pH 11 is the one that best explains the grouping of the aldehydes studied here. As for the ketone derivatives, they were grouped in the upper region of the scores diagram, which can be explained by analyzing the loadings diagram, because the variable TCC-pH10 accounts for this category of derivatives. Thus, the FCC-pH10 form is the one that best justifies the grouping of the ketone compounds studied. **Table 2** shows selected autoscores and their respective contributions to PC1 and PC2. **Table 3** indicates the accumulated percentage variance of calibrations and validation for PC1 and PC2 and how much each variable contributes to each principal component.

According to **Table 2**, components PC1 and PC2 can be expressed by the following equations:

$$PC1 = +0.599[BCC\text{-}pH11] + 0.588[BCC\text{-}pH10] + 0.544[TCC\text{-}pH10] \quad (7)$$

$$PC2 = -0.298[BCC\text{-}pH11] - 0.467[BCC\text{-}pH10] + 0.832[TCC\text{-}pH10] \quad (8)$$

In eq 7, the variables BCC-pH11 and BCC-pH10 are the ones that most contribute to separate the saturated (aldehydes and ketones) from the unsaturated carbonyl derivatives (alkenals). Of the three selected variables, BCC-pH11 is the one that most contributes to PC1. Indeed, this can be observed from the loadings diagram in **Figure 3** as well as from the fact all variables contribute positively to PC1. By analyzing eq 8, variables BCC-pH11 and BCC-pH10 are the ones that most contribute to the grouping of the aldehyde carbonyls, whereas the variable TCC-pH10 is the one that most contributes to the grouping of the ketone carbonyls. Thus, a detailed analysis of the scores and loadings diagrams reveals that the form bound at pH 11 is the one that is most interesting for the aldehydes and that the variable TCC-pH10 is the most interesting one for the ketones. **Table 3** indicates accumulated percentage variance. The proposed modeling of $\%Var_{accumulated}$ for calibration and validation is 99.60 and 98.46%, respectively. The $\%Var_{accumulated}$ for calibration indicates the model capacity to explain original data, showing as well its fitting power, whereas $\%Var_{accumulated}$ indicates the prediction capacity of proposed modeling. These results reveal that the BCC-pH11 variable is best adjusted as well as the most predictable in the model because it explains 96.78% and validates 94.62% of original data set just using the first principal component. The second principal component is necessary because it complements the analysis, showing the division between aldehydes and ketones. This also can be proved by data from **Table 3**, where the BCC-pH10 and TCC-pH10 variables should also be in the modeling because they contribute to validation of PC2. In this

Table 7. Concentrations (Milligrams per Liter, ± Standard Deviation) of Free, Bound, and Total CCs Identified in Five Different White Wines[a]

	wine 1		wine 2		wine 3		wine 4		wine 5	
CC	FCC	BCC	FCC	BCC	FCC	BCC	FCC	BCC	FCC	BCC
formaldehyde	3.50 ± 0,32	1.49 ± 0.85	5.23 ± 0.17	0.29 ± 0.15	2.45 ± 0.08	0.42 ± 0.22	5.61 ± 0.58	2.10 ± 0.46	14.13 ± 0.51	0.40 ± 0.17
acetaldehyde	1.68 ± 0.50	19.23 ± 0.37	2.14 ± 0.58	18.80 ± 0.44	5.68 ± 0.54	12.18 ± 0.61	1.12 ± 0.29	19.05 ± 1.05	4.60 ± 0.30	16.15 ± 0.57
furfural	1.87 ± 0.16	1.74 ± 0.17	4.58 ± 0.63	3.56 ± 0.28	5.79 ± 0.09	0.58 ± 0.04	4.34 ± 0.52	12.02 ± 0.61	2.52 ± 0.12	1.00 ± 0.22
butanal	8.14 ± 0.63	3.58 ± 0.85	2.32 ± 0.23	2.00 ± 0.13	1.62 ± 0.04	1.78 ± 0.47	6.23 ± 0.29	8.29 ± 0.28	1.84 ± 0.18	0.27 ± 0.03
benzaldehyde	1.72 ± 0.55	1.01 ± 0.22	1.79 ± 0.55	3.56 ± 0.66	8.59 ± 0.61	1.77 ± 0.59	3.04 ± 0.31	3.31 ± 0.24	0.51 ± 0.14	2.55 ± 0.80
hexanal	1.44 ± 0.48	0.90 ± 0.32	0.40 ± 0.14	1.60 ± 0.23	0.25 ± 0.12	1.30 ± 0.35	1.18 ± 0.40	0.59 ± 0.31	1.24 ± 0.29	0.41 ± 0.26
2-ethylhexanal	0.93 ± 0.04	1.00 ± 0.04	0.35 ± 0.03	1.60 ± 0.08	0.07 ± 0.003	1.50 ± 0.03	0.14 ± 0.06	1.20 ± 0.34	0.93 ± 024	1.31 ± 0.86

[a] FCC, free; BCC, bound.

Table 8. Concentrations (Milligrams per Liter, ± Standard Deviation) of Free, Bound, and Total CCs Identified in Five Different Red Wines[a]

	wine 1		wine 2		wine 3		wine 4		wine 5	
CC	FCC	BCC	FCC	BCC	FCC	BCC	FCC	BCC	FCC	BCC
formaldehyde	12.80 ± 0,32	0.81 ± 0.22	2.76 ± 0.26	0.51 ± 0.23	2.61 ± 0.31	0.91 ± 0.43	13.91 ± 0.50	0.53 ± 0.05	4.20 ± 0.22	0.32 ± 0.11
acetaldehyde	1.55 ± 0.45	1.35 ± 0.94	1.99 ± 0.17	3.14 ± 0.56	1.45 ± 0.12	0.94 ± 0.48	0.00 ± 0.00	5.25 ± 0.09	2.25 ± 0.66	5.44 ± 0.66
furfural	1.97 ± 0.30	1.15 ± 0.50	2.69 ± 0.42	0.62 ± 0.05	1.95 ± 0.22	0.84 ± 0.18	1.67 ± 0.18	1.08 ± 0.27	1.69 ± 0.34	5.65 ± 0.92
butanal	0.90 ± 0.19	0.49 ± 0.04	1.81 ± 0.26	1.08 ± 0.49	0.83 ± 0.14	0.91 ± 0.10	1.35 ± 0.04	1.15 ± 0.17	1.19 ± 0.14	6.15 ± 0.74
benzaldehyde	0.65 ± 0.08	0.60 ± 0.08	0.42 ± 0.11	0.10 ± 0.03	0.49 ± 0.06	1.38 ± 0.27	0.15 ± 0.008	0.07 ± 0.03	0.50 ± 0.08	0.80 ± 0.14
hexanal	0.16 ± 0.006	0.08 ± 0.03	0.25 ± 0.11	0.14 ± 0.03	0.09 ± 0.03	0.04 ± 0.004	1.54 ± 0.37	0.36 ± 0.16	0.15 ± 0.05	1.34 ± 0.29
2-ethylhexanal	0.15 ± 0.008	0.21 ± 0.02	0.17 ± 0.04	0.04 ± 0.004	0.09 ± 0.02	0.16 ± 0.06	0.36 ± 0.13	1.31 ± 0.37	0.18 ± 0.07	1.31 ± 0.17

[a] FCC, free; BCC, bound.

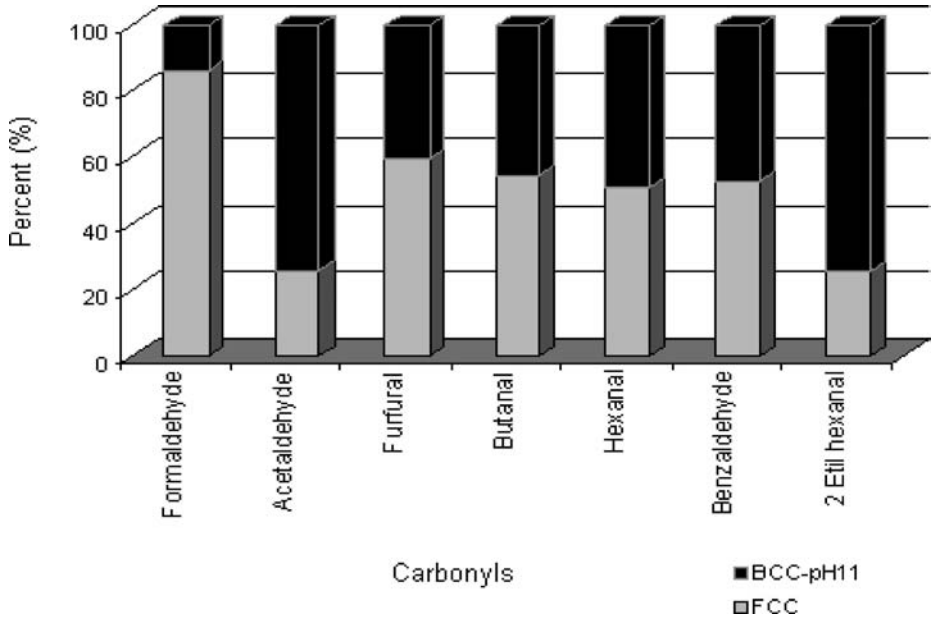


Figure 10. Average percentage of FCC and BCC compositions in 10 samples of real wines.

way, the BCC-pH11 variable is the one that best explains the original data set and best divides the sample groupings and discriminates categories, whereas BCC-pH10 and TCC-pH10 most contribute to a good discrimination of categories by functional groups of both aldehydes and ketones.

Tables 4 and **5** list the values of the concentrations of free, bound, and total carbonyl compounds in "synthetic wine", analyzed individually and jointly, respectively. The values of FCC are equivalent to the results found in the sample contained in flask B1 (**Figure 2**), whereas the values of TCC-pH 9–13 are equivalent to the results found in flasks C1–C5, respectively. Concentration values of samples from flask A1 (bisulfite blank, BB) were used as reference in the calculations of FCC and TCC. Taking into account that in the preparation of A1 an aliquot of 1 mL of 10 mg L^{-1} of CC was diluted into 5 mL of 2,4-DNPH for derivatization reaction, then in all cases the final concentration of CC in A1 was 1.67 mg L^{-1}.

The data indicate that adduct formation does not appear to be conditioned only on the presence of carbonyl in the organic compounds studied. The values of the FCC and BCC concentrations demonstrate that the position of the carbonyl group, the size of the chain, and the trans character of the structure of the stereoisomers of CCs can influence the formation of the BCC.

The graphs depicted in **Figures 6** and **7** show the free and bound fractions of the CCs after the reaction with bisulfite, in synthetic wine. The data of FCC and BCC, expressed in percent, were calculated on the basis of TCC, assumed as 100%, except for the *trans*-alkenals, the FCC values of which needed to be calculated on the basis of the value of the blank (A1); therefore, the determination in the total form presented problems and will be argued ahead.

All values of BCC refer to the analysis in which pH 11 was used in the dissociation of the adduct, because this proved to be the most appropriate pH. **Figure 6** illustrates the profile of

the CC studied individually, whereas **Figure 7** represents the profile of the 22 CCs mixed together. Both graphs indicate that the stability of the BCC is greater for the saturated aliphatic carbonyl compounds (formaldehyde, acetaldehyde, propanal, pentanal, hexanal, octanal, nonanal, and decanal) and that, in most cases, >50% of the concentration of these compounds is present in bound form. In the individual study of these compounds, the BCC values are even higher. Among them, the one displaying the strongest interaction with bisulfite is formaldehyde, an aldehyde with a very short chain containing only one carbon atom. Approximately 90% of the formaldehyde was present in bound form. As for the benzene aldehydes (benzaldehyde and salicylaldehyde), they do not form adducts in significant quantities.

When studied separately, the ketones also do not display a strong affinity for the bisulfite ion, because significant contents of the BCC formed from this group were not identified, except for cyclopentatone, with 20% of its concentration in the combined form. Surprisingly, when evaluated in the mixture, the ketones were found to be, on average, 10% combined in adduct form.

For the alkenals, the lack of graphic representation for BCC values, shown in **Figures 6** and **7**, is due to the difficulty in determining the total contents of these components by the method proposed in this work. Two mechanisms are suggested to explain this phenomenon. One of them, the alkaline medium required in TCC quantification, becomes much easier than the simple addition reactions between α- and β-unsaturated aldehydes or ketones and strong nucleophilic species such as hydroxide ion (*35*). In this way, the hydroxide ion will be added to unsaturated CC through the carbonyl double bond (**Figure 8**), resulting in alcohols or other compounds unidentified by this method. The other mechanism could be related to the conjugated addition reaction between anions and α- and β-unsaturated CCs, known as "Michael addition" (**Figure 9**). In this case, bisulfite would be added to the 1,4-position in relation to the carbonyl group, removing the double bond of the carbon chain without changing the carbonyl groupl; the new compound will be attacked by hydroxide ion, giving a different adduct. This adduct would not be able to react with 2,4-DNPH to form the hydrazone, and the analytical signal could not be obtained. Therefore, new studies are ongoing to solve this problem.

The dissociation constants (K_d) for several carbonyl bisulfite compounds evaluated here were calculated according to eq 3, using free S(IV) species values determined by volumetric titration with iodine, free carbonyl compound molar concentrations determined by chromatographic method, and bound carbonyl compound molar concentrations obtained by the difference between total and free carbonyl compounds. The dissociation constant values obtained for each carbonyl compound at pH 3.5 are found in **Table 6**. The values are in agreement with literature data for formaldehyde (*28*) and acetaldehyde (*15*, *16*, *37*) and match the trend of adduct stability foreseen by PCA; that is, the K_d values for aliphatic aldehyde adducts are smaller than for ketones, cyclic aldehydes, and *trans*-alkenes adducts.

The average concentration values of FCC and BCC found for real samples (white and red wines) when this method was applied are summarized in **Tables 7** and **8**, whereas the percentage compositions of FCC and BCC are shown in **Figure 10**.

Among all CCs identified in real wines, formaldehyde and furfural are the only ones present predominantly in free form, 86.21 and 59.53%, respectively, whereas acetaldehyde and 2-ethylhexanal prevail in bound form, 74.00 and 74.36%, respectively. Butanal, benzaldehyde, and hexanal are found to be uniformly distributed as free and bound forms. When analyzed together in a "synthetic wine", formaldehyde, acetaldehyde, benzaldehyde, and hexanal showed the same profile.

Although butanal was absent in synthetic wine, we could identify this CC in real wine samples, and its profile is quite similar to the aliphatic aldehyde with three to eight carbon atoms in the synthetic wine. The same profile was observed for furfural, which resembles benzaldehyde, an aromatic aldehyde. Both CCs showed just a little affinity for bisulfite ion, being present in higher concentrations in their FCC forms in real wines.

In conclusion, evaluation of the 22 carbonyl compounds in sulfited synthetic wine and of several of them in real wine samples, therefore, revealed that the molecular structure of these aldehydes and ketones plays a relevant role in BCC formation, because aliphatic aldehydes were found to constitute the CC group with the highest affinity for bisulfite (~50% of their concentration is represented in bound form). On the other hand, for the ketones, the percentage of the BCC fraction is very small in relation to the aldehydes having chains that possess the same number of carbon atoms. Both the aliphatic and cyclic ketones were predominantly present in their free form.

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