JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Determination of 2,3-Butanediol and 2-Hydroxybutanone Stereoisomers in Batteries of Traditional Balsamic Vinegar

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The absolute quantities and the stereoisomeric ratios of *R*,*S*-2-hydroxybutanone and *R*,*R*-, *S*,*S*-, *R*,*S* (*meso*)-2,3-butanediol were determined in batteries of traditional balsamic vinegar (TBV) by gas chromatography–mass spectrometry (GC–MS), using a chiral capillary column, to evaluate if such parameters could be used to differentiate TBV differently aged and from different producers. Results showed that the initial amounts of 2-hydroxybutanone and 2,3-butanediol were quite variable, as a function of the producer of the vinegar; moreover, the 2-hydroxybutanone amount decreased during aging while 2,3-butanediol increased. Initially, the *R*-2-hydroxybutanone form prevails, and then the *R*/*S* ratio decreased regularly during aging with some exceptions attributed to the addition of new barrels during the battery management. With regard to the 2,3-butanediol isomers, the most abundant was the *R*,*R* form, slowly transformed into the *R*,*S* and *S*,*S* isomers during aging. The GC–MS method used is easy and fast and could allow for a quick control of the maturation level of the vinegar.

KEYWORDS: 2-Hydroxybutanone; 2,3-butanediol; traditional balsamic vinegar; stereoisomers; GC–MS

INTRODUCTION

The traditional balsamic vinegar (TBV) of Modena and the traditional balsamic vinegar of Reggio Emilia received, on May 15, 2000, the protected denomination of origin (PDO) certification from the European Union (*1*), because of their typical production procedures and the well-defined geographical areas of their production.

From their regulations (*2*), which are very similar, the TBVs have to be produced only in Modena and Reggio Emilia, Italian provinces, using must from species of vine traditionally cultivated in these areas. The most used vine is Trebbiano, because it reaches the best composition at the optimal ripening stage (ratio of sugars/total titrable acidity). The must is concentrated by gently simmering over an open fire in uncovered pans. The must is then put in characteristic sets of wooden barrels of different volumes (batteries) for the aging. The batteries are composed by barrels of decreasing capacity, usually in a number of 5. Anyhow, the best vinegar refinement conditions are obtained using 7–10 barrels of different kinds of wood: mulberry, cherry, chestnut, oak, and juniper. Each kind of wood gives the product particular substances responsible for its "bouquet". Traditionally, the set of barrels is located in the garret of the vinegar factory, because the high temperature during the summer promotes microbial activity, while the low temperature during winter favors clarification and flavor evolution. During the aging process that is carried out for a period not shorter than 12 years, the level of the liquid in each barrel is kept constant ($^2/_3$ of the barrel volume) by transferring periodically a certain amount of vinegar from one barrel to another, in a decreasing progression, while in the first barrel, fresh must is added. This procedure is called "topping up". In the course of the first stage of aging, there is an intense microbial activity, first because of the growth of osmofile yeasts (*Zygosaccharomyces*), corresponding to ethanol production, and second to *Acetobacters* that transform ethanol into acetic acid and inhibit the growth of yeasts (*3*). Microbial activity is often limited to the first barrel, while inside the others, TBV modifications are due to chemical and biochemical reactions. TBV ripening is in fact accompanied by a series of biochemical reactions that produce a complex mixture of compounds, responsible for its typical flavor. Many studies were performed in the last 30 years about the characterization of TBV, aimed at determinating the trend of specific substances during aging, e.g., carboxylic acids (*4*, *5*), sugars (*6*, *7*), amino acids (*8*), and furanic compounds (*9*), but many aspects of the chemical modifications that occur in TBV, mainly regarding minor compounds, remain unknown.

2-Hydroxybutanone (acetylmethylcarbinol or acetoin) and 2,3-butanediols are chiral compounds and exist, respectively, in two (*R* and *S*) and three (*R*,*R*, *S*,*S*, and *R*,*S* or *meso*) stereoisomeric forms.

2-Hydroxybutanone is produced during the alcoholic fermentation by action of several microrganisms, and it is present in fermented foods and beverages, such as wine,

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10.1021/jf071206n CCC: $37.00
Published on Web 08/22/2007

Table 1. Absolute Quantities and Stereoisomeric Composition for 2-Hydroxybutanone and 2,3-Butanediol

sample name	barrel volume (L)	year	°Brix	2-hydroxybutanone (mg kg^{-1})	2,3-butanediols (mg kg^{-1})	2-hydroxybutanone (*R*/*S*)	2,3-but (% *S*,*S*)	2,3-but (% *R*,*R*)	2,3-but (% *R*,*S*)
A1[a]	75	1993	34.3	165	110	1.45	12.5	49	38.5
A2[a]	60	1993	33.3	134	177	1.54	8.1	51	40.9
A3[a]	50	1993	39.0	169	208	1.70	6.6	60	33.4
A4	40	1974	48.3	87	292	1.88	3.4	66	30.6
A5	30	1974	62.4	60	831	1.74	3.8	66	30.2
A6	25	1974	72.5	26	1135	1.68	3.6	65	31.4
A7	20	1974	74.2	14	1250	1.52	4.5	62	33.5
A8	15	1974	73.3	23	1428	1.50	5.4	61	33.6
B1[a,b]	150	1990	26.0	149	328	1.53	7.1	63	29.9
B2	60	1976	45.7	135	129	1.89	4.3	62	33.7
B3	40	1976	49.5	84	261	1.77	4.7	64	31.3
B4	30	1976	52.7	76	358	1.68	5.2	62	32.8
B5	20	1976	56.0	77	467	1.42	7.2	59	33.8
B6	15	1976	59.0	79	526	1.25	7.9	58	34.1
C1[a,b]	225	1980	22.5	505	940	2.02	22	32	46
C2[a,b]	225	1980	27.3	286	575	2.05	15.5	39	45.5
C3	50	1970	50.5	179	464	1.90	6.9	52	41.1
C4	40	1970	55.0	151	511	1.40	6.5	58	35.5
C5	30	1970	63.1	123	598	1.42	4.2	63	32.8
C6	25	1970	67.5	52	646	1.40	4.2	61	34.8
C7	20	1970	72.8	25	675	1.31	4.9	60	35.1
C8	15	1970	70.5	34	653	1.40	4.8	60	35.2
D1	60	1975	40.5	300	626	3.53	7.6	59	33.4
D2	50	1975	42.6	325	652	3.18	7.9	58	34.1
D3	40	1975	45.6	289	725	2.93	8.1	56	35.9
D4	35	1975	49.2	290	904	2.57	9	53	38
D5	30	1975	53.5	207	1109	2.31	10	51	39
D6	25	1975	52.2	191	1112	2.11	10.8	48	41.2
D7	20	1975	59.5	201	1361	1.85	11.7	47	41.2
D8	10	1975	61.2	203	1421	1.79	11.8	47	41.2
D9	5	1975	64.7	155	1561	1.59	12.9	46	41.1

[a] Barrels added later to the battery. [b] Barrels with °Brix lower than 30.

vinegar, and several dairy products; the metabolic pathway requires condensation and decarboxylation of two molecules of pyruvic acid, but it can also derive from the spontaneous degradation of acetolactate (*10*). The 2,3-butanediol is a volatile compound contributing to the flavor of various foods; it is a secondary product of the alcoholic fermentation and derives from the reduction of 2-hydroxybutanone (*11*). 2-Hydroxybutanone in fresh wine and vinegars is mainly present in the natural more abundant *R* form, while 2,3-butanediol is mainly present in the *R*,*R* form (*12*). The production of 2-hydroxybutanone and 2,3-butanediol in wine and their enantiomeric ratios may be connected to the yeast genus involved in the fermentation (*13*, *14*).

Only a few data about 2-hydroxybutanone and 2,3-butanediol in TBV can be found in the literature: it was shown (*15*) that the 2,3-butanediol amount in TBV is higher than the 2-hydroxybutanone content; moreover, TBV contained higher values of both of the alcohols with respect to the other typologies of the vinegar (balsamic vinegar and wine vinegar), owing to the vinegar concentration effect (*16*).

The *R*/*S* ratio of 2-hydroxybutanone was previously determined in TBV and balsamic vinegar (*17*), allowing for the discrimination between the two vinegar typologies.

To our knowledge, no studies were carried out to determinate the stereoisomeric composition of 2,3-butanediol in TBV. Therefore, the main aim of the present work was to obtain deeper information about the 2,3-butanediol and 2-hydroxybutanone contents and their stereoisomeric changes during the aging of TBV.

MATERIALS AND METHODS

Materials. *R*,*S*-2-Hydroxybutanone, 2,3-butanediol stereoisomers (*R*,*R*, *S*,*S*, and *meso*), and 1,4-butanediol were purchased from Sigma-Aldrich (Milan, Italy); reagents and solvents (ethyl acetate, sodium bicarbonate, and glacial acetic acid) were purchased from Carlo Erba Reagenti (Milan, Italy).

Sampling. TBV samples were supplied by four different producers from Modena and Reggio Emilia areas. In the batteries (named A, B, C, and D), samples were collected from each barrel (corresponding, approximately, to vinegars aged between 0 and 25 years). The barrel capacities in each battery are reported in **Table 1**. In the text, samples will be abbreviated with the name of the battery and a progressive number from the younger to the oldest sample. In more details, battery A was started in 1974, and it was composed of five barrels (A4, A5, A6, A7, and A8); the other three barrels (A1, A2, and A3) were added in 1993: the barrels A1 and A2 were filled with cooked must, while the A3 barrel was filled with a mixture of TBV of medium ages. Battery B was started in 1976, and it was initially composed of five barrels (B2–B6). The last barrel (B1) was added later (1990) and was considered external to the battery; it was refilled every year with newly produced cooked must and wine vinegar. Battery C, initially composed of six barrels (C3–C8), was started in 1970; barrels C1 and C2 were added later (1980) and, similar to B1, were external to the battery and filled with a blend of cooked must and wine vinegar. Battery D was started in 1975; it is composed of 9 barrels, and the "topping up" operations were conducted regularly.

Sample Treatment for the Analysis of 2-Hydroxybutanone and 2,3-Butanediol. The soluble dry matter content (°Brix) of the vinegars was determined by refractometry at 20 °C, after dilution 1:1 (w/w) of the thick samples with distilled water. All of the vinegars (except C1) were diluted to 25 °Brix with distilled water to perform the following

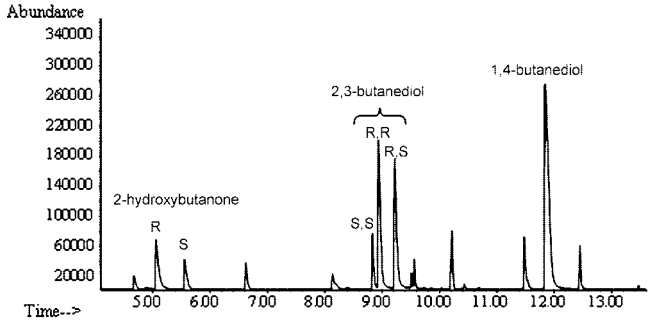

Figure 1. GC–MS chiral resolution of 2-hydroxybutanone and 2,3-butanediol stereoisomers in a TBV sample.

analyses. 2-Hydroxybutanone and 2,3-butanediol were determined according to a method previously reported for wine (*14*), adapted to vinegar analysis: 1 mL of internal standard (1,4-butanediol, 1000 ppm in water with 6% acetic acid) was added to 0.5 mL of the diluted vinegar; the samples were neutralized by the addition of solid $NaHCO_3$ and extracted twice with 2 mL of ethyl acetate. The organic phase was concentrated to 0.2 mL under nitrogen flow and injected in gas chromatography–mass spectrometry (GC–MS) .

GC–MS analysis was performed with an Agilent Technologies 6890N gas chromatograph coupled to an 5973N mass selective detector (Agilent Technologies, Santa Clara, CA), under the following instrumental conditions: CP Chirasil-Dex capillary column (25 m, 0.25 mm i.d., 0.25 μm film thickness; Chrompack) with the oven temperature programmed from 50 °C for 3 min and then increased to 160 °C at 10 °C/min. The head pressure was 6.93 psi, with injector temperature, 230 °C; injection mode, split; volume injected, 1 μL; detector temperature, 230 °C; carrier gas, helium. MS conditions: ion source temperature, 230 °C; electron impact, 70 eV; acquisition mode, SIM (*m*/*z* 42, 45, 57, 59, and 72). A typical GC-MS chromatogram obtained for a TBV sample is reported in **Figure 1**. The elution order of 2,3-butanediol stereoisomers was determined by comparing retention times of analytes in the sample with those of the three optically pure stereoisomers. In the case of 2-hydroxybutanone, only the standard racemic mixture was disposable; therefore, the identification of the first eluted peak as *R*-2-hydroxybutanone was made on the basis of its predominant natural abundance in alcoholic fermented media (*12*) and on the well-known elution order of 2-hydroxybutanone enantiomers on permethylated β-cyclodextrin stationary phases (*18*, *19*).

Determination of Response Factors (RFs). 2-Hydroxybutanone and 2,3-butanediol were quantified by means of the internal standard (1,4-butanediol) that allows us to calculate the RF, according to the following equation:

$$RF_i = \frac{C_i}{C_{st}}\frac{A_{st}}{A_i} \quad (1)$$

where RF_i is the response factor for the *i*th species, C_i is the concentration (mg L^{-1}) of the *i*th species, C_{st} is the concentration (mg L^{-1}) of the internal standard, A_i is the peak area of the *i*th species, and A_{st} is the peak area of the internal standard.

The standard solution used for the calculation of RF_i was prepared dissolving 2-hydroxybutanone, 2,3-butanediol, and 1,4-butanediol (exactly known amounts) in a solution of water with 6% acetic acid, to simulate the real conditions of the analytes in the vinegar samples. The solution was subjected to the same analytical procedure as the samples.

Reproducibility. The same standard solution was also used for determining the instrumental interday reproducibility: the standard sample was analysed every 3 days for a period of 20 days; on each day, three repetitions of the analysis were performed. The data were treated with one-way analysis of variance (ANOVA) .

Determination of Linearity. The linearity of the method was determined in the concentration intervals of 10–500 ppm for 2-hydroxybutanone and 100–1500 ppm for 2,3-butanediol. In each interval, six concentrations (10, 20, 50, 100, 200, and 500 ppm for 2-hydroxybutanone and 100, 200, 300, 500, 1000, and 1500 ppm for 2,3-butanediol) were considered. Each solution was prepared in water with 6% acetic acid, added with the same quantity of 1,4-butanediol (1000 ppm, 1 mL), and subjected to the same treatment as the samples. Three replicates were performed for each concentration. A regression was performed on the ratio of peak areas of analyte and internal standard versus the analyte concentration.

Recovery. Recovery was determined first by comparing the analytical results for extracted standards at three concentrations (50, 500, and 1000 ppm of both 2-hydroxybutanone and 2,3-butanediol) with unextracted standards and after by an addition method. In the last case, a medium-aged vinegar (D4) was spiked with known amounts of analytes (50, 500, and 1000 ppm). The spiked and nonspiked vinegars were analysed in triplicate following the proposed method, and their concentrations were calculated by the internal standard. The recovery was estimated as

recovery (%) = ((calculated concentration in spiked vinegar − calculated concentration in the nonspiked vinegar)/ concentration added) × 100

Repeatability. The repeatability of the GC–MS method was determined by nested ANOVA. The test was done according to the following experimental design: two different analysts operated three different extractions on the same vinegar sample according to the experimental procedure, and each extract was injected 3 times in the GC–MS system. The analysis of variance method was used to determine the statistical significance of the fixed effects: analyst, extraction, and GC–MS analysis by the statistical *F* test using the integration as an error term. The tests were carried out at the 0.05 significance level using the data obtained from the signal integrations referred to 1,4-butanediol.

Statistical Methods. ANOVA and principal component analysis (PCA) were performed using SPSS 12.1 software.

RESULTS

2-Hydroxybutanone and 2,3-butanediol were quantified by means of the internal standard method, as reported in the Materials and Methods. The RFs calculated (GC–MS analysis in SIM mode) were 0.137 ± 0.003 for 2-hydroxybutanone and 0.228 ± 0.006 for 2,3-butanediol. The RFs for the two 2-hydroxybutanone enantiomers and the three 2,3-butanediol stereoisomers do not differ significantly; therefore, all of the following analytical parameters can be referred indifferently to the single stereoisomer or the racemic mixture. The linearity was demonstrated by means of the fundamental calibration in the range of 10–500 ppm for 2-hydroxybutanone ($R^2 = 0.9938$ per $n = 18$) and in the range of 100–1500 ppm for 2,3-butanediol ($R^2 = 0.9986$ per $n = 18$). The linearity in the range of the sample concentration also demonstrates the constancy of the RF in the same interval.

The influence of the extraction procedure on the recovery was determined comparing the peak areas of the analytes, referred to the internal standard, in the standard solution before and after the solvent extraction. The recoveries were good (over 95%) if, during the sample concentration under nitrogen flow, the complete desiccation of the sample was avoided. The recovery from the TBV samples was also determined by the standard addition method, to verify the presence of matrix effects. A TBV of medium aging was analyzed first and after spiking with solutions at three different concentrations of the analytes, and the percent of recovery was determined for each concentration, as reported in the Materials and Methods. The average recovery was 94 ± 1% for 2-hydroxybutanone and 97 ± 2% for 2,3-butanediol.

The evaluation of the interday reproducibility, by means of the one-way ANOVA test, showed that no significant differences (p level = 0.05) had arisen in the time interval under study.

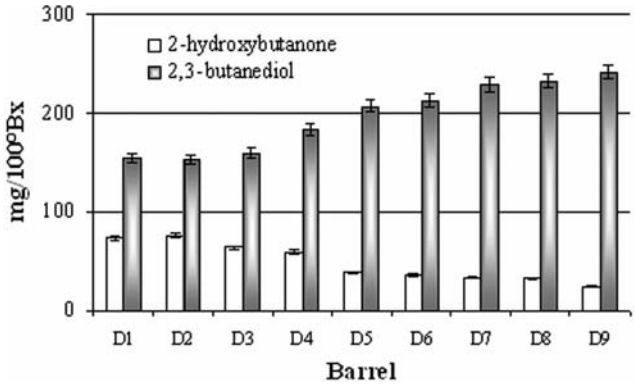

Figure 2. Variations of the absolute amounts of 2-hydroxybutanone and 2,3-butanediol referred to soluble refractometric dry matter (mg/100 °Brix), during the aging of TBV in battery D.

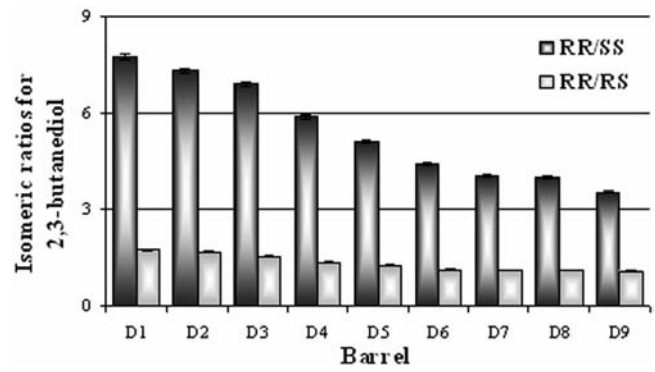

Figure 3. Trend of 2,3-butanediol stereoisomer ratios (*R,R*/*S,S* and *R,R*/*R,S*) during the aging of the battery D samples.

The reproducibility of the whole method was determined by the variance analysis with hierarchical classification of parameters (nested ANOVA). The test showed that all of the variance components of the parameters considered (analyst, extraction, and GC–MS analysis) were not statistically significant (p level 0.05), confirming a satisfactory reproducibility for the method. The overall standard deviation % (SD %), obtained from the reproducibility study, was less than 3.5% for 2-hydroxybutanone and less than 3% for 2,3-butanediol quantification. Better standard deviations (about 1%) were obtained for the stereoisomeric ratios.

The data collected for the vinegars of all four batteries are summarized in **Table 1**, which reports the absolute quantities (mg kg^{-1}) of 2-hydroxybutanone and 2,3-butanediols together with their stereoisomeric composition. For 2-hydroxybutanone, the *R*/*S* ratio is reported, while for 2,3-butanediols, the percentage values of *R,R*, *S,S*, and *R,S* (*meso*) forms are shown. From the results reported in **Table 1**, it arises that the initial absolute quantities of both 2-hydroxybutanone and 2,3-butanediol are quite variable as a function of the battery. This can be related to the natural microorganism pattern involved in the fermentation processes, which may be slightly different for each battery (*20*). The initial amount of 2-hydroxybutanone tends to decrease regularly in all of the batteries during aging, while the quantity of 2,3-butanediol increases. The slow transformation of 2,3-butanediol into 2-hydroxybutanone can be partially responsible for these variations. In batteries B and C, the increasing trend of 2,3-butanediol is respected if we exclude, respectively, the barrel B1 and the barrels C1 and C2. These barrels contain a blend of cooked must and wine vinegar, which can explain both their low soluble dry matter content (°Brix) and their anomalous behavior with respect to the trend observed in the other barrels.

To verify whether the trends recorded were dependent upon the natural TBV concentration occurring during aging, the absolute quantities of the analytes were also referred to soluble dry matter content (°Brix). The results confirmed that the trends observed for 2-hydroxybutanone and 2,3-butanediol amounts are effectively due to chemical or biochemical transformations, because they are also maintained when data are expressed as a function of °Brix (**Figure 2**).

With regard to the stereoisomeric composition of 2-hydroxybutanone, the richer isomer in TBV is always the *R* form, as detected in wines (*12*). The trend of the *R*/*S* ratio during aging is a regular decrease in all batteries if we consider the barrels with the same age, while the barrels added later show values not always in agreement with the battery trend.

The decreasing trend for the *R*/*S* value of 2-hydroxybutanone in TBV during aging is in good agreement with data previously reported (*17*).

It is interesting to note that the battery in which all of the barrels were started simultaneously (battery D) shows a more regular trend than those in which new barrels were added (A, B, and C). Therefore, the control of the *R*/*S* ratio of 2-hydroxybutanone during aging could give evidence of possible irregular managements of the "topping up" operations in the TBV factory.

The initial stereoisomeric distribution of 2,3-butanediol has, in general, the *R,R* form as prevalent isomers, followed by *R,S* and minor amounts of the *S,S* isomer. This isomeric composition is compatible with an alcoholic fermentation carried out by *Saccharomyces* species, which, as previously reported (*14*), gives in the fermenting medium an isomeric composition for 2,3-butanediol of 79% *R,R*, 20% *meso*, and 1% *S,S*. Barrels C1 and C2 represent an exception, because they contain the *R,S* (*meso*) form as a prevalent isomer. The prevalence of the *meso* form can be related to other wild species of yeasts (for example, *Kloeckera*, *Candida*, *Hanseniaspora*, and *Metschnikowia*). The distribution of 2,3-butanediol stereoisomers gradually changes during the aging period, because the most abundant isomer, *R,R*, is slowly isomerized to the *R,S* and then to the *S,S* forms, with a decrease of the isomeric ratios (*R,R*/*S,S* and *R,R*/*R,S*), as shown in **Figure 3** for battery D. In battery A, this trend is confirmed if we exclude the barrels added later (A1, A2, and A3) that were filled with a blend of different products. The behavior of battery C is more difficult to explain: we can make the hypothesis that the different products contained in barrel C1 and C2 had progressively modified the original isomeric composition of the battery. In fact, the trend of 2,3-butanediol stereoisomers became regular starting from barrel C5.

To better understand the data obtained in this work, a multivariate PCA was performed, considering as variables the total amount of 2-hydroxybutanone and 2,3-butanediol and their stereoisomeric composition. PCA was performed on a matrix of six variables measured in all of the 31 TBV samples, using the correlation matrix. From the PCA results, the first three components were significant, explaining, respectively, 59.5, 19.4, and 15.2% of the system variance that altogether amounts to 94% of the total variance. The scatter plots of the score of the samples on PC1 versus PC2 and on PC1 versus PC3 are shown in **Figure 4**.

The graph of PC1 versus PC2 (**Figure 4a**) does not show very evident groupings; however, it is interesting to notice that, except for battery C (whose behavior was influenced by the anomalous composition of barrels C1 and C2), most aged samples of each battery (B6, A8, and D9) are discriminated

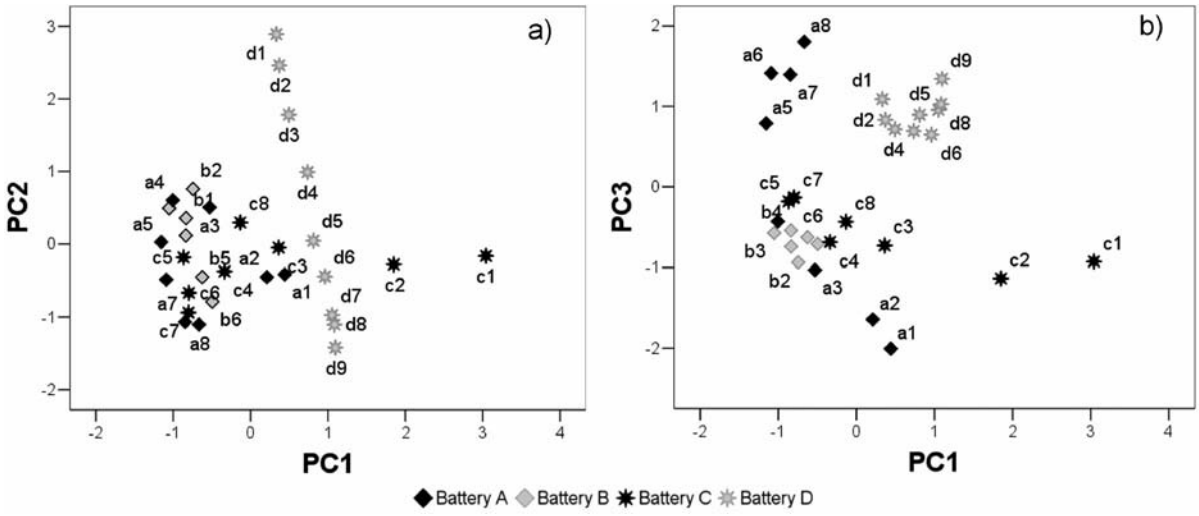

Figure 4. Scatter plot of the scores of TBV samples on (a) PC1 versus PC2 and (b) PC1 versus PC3.

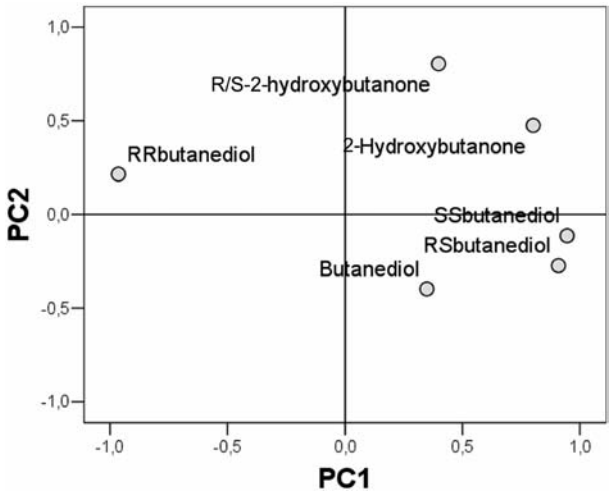

Figure 5. Loadings of the variables on PC1 and PC2.

along PC2. PC1 also determines a slight separation of the sample from different batteries. When PC1 is plotted against PC3 (**Figure 4b**), a better grouping according to the battery can be evidenced: battery D is well-separated from the others, demonstrating that a regular management of the battery influences the product composition; battery B forms a definite group, while for battery A and C, the grouping is evident only for the oldest barrels that are less influenced by the new barrel addiction. From the loading values of the variables associated to the first two principal components reported in **Figure 5**, it arises that stereoisomeric composition of 2,3-butanediol has the most significant effect on PC1, the principal component that mainly discriminates among TBV from different producers; this is in agreement with the observation that 2,3-butanediol stereoisomeric composition can be related to the indigenous microflora of the specific battery. 2-Hydroxybutanone amounts have a significant value only on PC1. The *R*/*S* value of 2-hydroxybutanone shows the highest value on PC2, the component associated to the discrimination according to age, confirming that the *R*/*S* ratio of 2-hydroxybutanone can be considered a good marker of the aging process. On PC3, only the 2,3-butanediol absolute quantity has a significant loading value.

On the basis of the preliminary data obtained in this work, we can conclude that chiral analysis of TBV components is useful to understand the modification occurring during aging. The application of the quantification method of 2-hydroxybutanone and 2,3-butanediol isomers to more numerous samples of TBV could give a contribution to the resolution of the TBV age problem.

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Received for review April 24, 2007. Revised manuscript received July 17, 2007. Accepted July 21, 2007.

JF071206N