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Modified Nondestructive Colorimetric Method To Evaluate the Variability of Oxygen Diffusion Rate through Wine Bottle Closures

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Some modifications to a previous nondestructive colorimetric method that permits evaluation of the oxygen diffusion rate through wine closures were proposed. The method is based on the reaction of indigo carmine solution with oxygen and the tristimulus measurement of the consequent color change. Simplified preparation and measurement procedures were set up, allowing the analysis of a large number of samples simultaneously. The method was applied to the evaluation of the variability within the lot of 20 different types of stoppers (synthetic, produced by molding, and natural cork). The closures were tested at a storage temperature of 26 °C. With regard to oxygen permeability, the natural cork stopper showed a low homogeneity within the lot, especially during the first month after bottling, whereas the synthetic closure showed a greater steadiness in the performance. The limits of the colorimetric method were also analyzed, and three possible causes of degradation of the indigo carmine solution were identified: oxygen, light, and heat.

KEYWORDS: Bottles; oxygen; wine; permeability; indigo carmine

INTRODUCTION

During recent years an increasing interest in the evolution of wine quality during postbottling storage has been observed. An important outcome of the research has been the statement that starting from the same wine, different products could result with bottling under different closures (*1*). In addition, the lack of homogeneity in wine development among different bottles using the same closure is a problem often observed by winemakers.

Greater variability in the performance of closures has been observed in natural cork. Due to inconstant origin of raw materials, cork stoppers often present structural, dimensional, and mechanical characteristics difficult to standardize (*2*); in particular, high standard deviation in mass and density (*3*, *4*), random phenomena of TCA pollution (*5*, *6*), highly variable oxidative spoilage that affects only a limited number of bottles from a bottling run (*7*−*9*), and large variations in SO_2 levels between different bottles (*10*, *11*) have been observed.

It appears clearly that the variables that influence postbottling storage are numerous and interlinked in complex relationships; one of the most important factors that influence the variation in wine development seems to be the oxygen permeation rate (*1*, *7*, *12*, *13*). The bottling is a critical point in the enrichment of wine with oxygen (*14*, *15*), and any additional amount of oxygen that the wine comes into contact with during bottled aging is determined by the ineffectiveness of the oxygen barrier created by the bottle closure (*10*). Several methods have been developed to quantify the rate of oxygen permeation through the closure, and they can be distinguished as destructive and nondestructive methods. Measurement of dissolved oxygen by polarographic probe (Orbishere), headspace analysis with a GC method, measurement of total oxygen in a bottle using an oximeter and the application of Henry's law (*16*) are destructive methods that do not permit the evolution of a single bottle to be followed over time. The Mocom method, based on the measurement of oxygen permeation rate, using a coulometric sensor (*1*, *17*), and the monitoring of oxygen migration by GC analysis (*12*), even if nondestructive, are very expensive, applicable only on a limited number of bottles, and allow measurement only in dry packaging, which does not reproduce wine aging (closure in contact with wine). Measurement of the absorbance at 420 nm on clear or colored bottles (*18*) and determination of acetic acid in wine bottles using high-resolution ^{1}H NMR spectroscopy (*19*), even if nondestructive, are always indirect evaluations of oxygen migration rate. The nondestructive colorimetric method, based on the color change from oxidation−reduction of indigo carmine (*20*−*22*), allows the kinetic study of oxygen entry through the closure over time, without compromising the closure seal, but it requires a long analysis and cannot provide multiple replicate tests for multiple closure types.

The published data about oxygen diffusion show that there is a great variability among different types of cork and synthetic stoppers (*10*, *17*, *20*, *21*), with very low rate for screw caps (*1*, *21*), but due to the reduced number of replicates used in these studies, there were no data regarding the variation among stoppers of the same lots.

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Table 1. Codes and Sizes of the Closure Types in the Experiment

closure	*n*	size (mm)	closure	*n*	size (mm)
cork A	10	44 × 24	synthetic A	10	40 × 23
cork B	10	44 × 24	synthetic B	10	39 × 23
cork C	10	44 × 24	synthetic C	10	39 × 23
cork D	10	44 × 24	synthetic D	10	44 × 21.5
cork E	10	44 × 24	synthetic E	10	37 × 23
cork F	10	44 × 24	synthetic F	10	43 × 23
cork G	10	44 × 24	synthetic G	10	37 × 23
cork H	10	44 × 24	synthetic H	10	42 × 22
cork I	10	44 × 24	synthetic I	10	39 × 21.5
cork J	10	44 × 24	synthetic J	10	37 × 23

Because variability in oxygen migration inside bottles could be strictly related to random oxidative spoilage, variations in SO_2 level, and the appearance of maderized flavors during bottle storage, the aim of this work is to propose a simplification of the previous colorimetric method to permit the evaluation of the oxygen permeation rate in a larger number of samples, without loss of accuracy. Particularly, we modified the calibration and measurement procedures to ease and speed the operations. The stability over time of indigo carmine solution has also been evaluated to understand for how long the kinetics of oxygen migration can be followed. The simplified method was then used to evaluate the variability among and within the lots of 10 natural cork stoppers and 10 synthetic stoppers.

MATERIALS AND METHODS

Reagents. Indigo carmine (for microscopy grade) and sodium benzoate (≥99.5%) were purchased from Fluka BioChemica (Buchs, Switzerland); sodium dithionite (≥86%) was purchased from Riedel de Haën GmbH (Seelze, Germany). Deionized water (conductivity = 7.5 μS/cm at 20 °C) purified with a Milli-Ro system (Millipore, Bedford, MA) was used to prepare solutions.

Bottles and Closures. Extra white bordelaise classic bottles (750 mL), with a diameter of 17.5 mm at a depth of 3 mm from the bottle entrance (Art. 530/B), were purchased from Triveneta Vetro Srl (Treviso, Italy). The same bottles were used for the calibration and measurement procedures. To evaluate the indigo carmine solution stability, Pyrex SVL bottles (100 mL) purchased from Vetrotecnica (Verona, Italy) were used. Twenty different kinds of closures were tested: 10 lots of first-grade natural cork stoppers and 10 lots of synthetic closures produced by a molding process (**Table 1**). The closures were provided by a range of suppliers representative of the current market situation.

Preparation of Indigo Carmine and Sodium Dithionite Solutions. The method to evaluate the oxygen permeation rate through closures is based on the color change from oxidation−reduction reaction of indigo carmine (*20*). An indigo carmine (0.25 g/L) and sodium benzoate (5 g/L) solution in deionized water was prepared to fill the bottles. The solution was homogenized very carefully and completely saturated with oxygen. Just before the bottling procedure was begun, 15 g of sodium dithionite was added to 1 L of deionized water. This solution was immediately closed with a bottle-top dispenser, Dispensette III 25 mL (Brand GmbH Wertheim, Germany), to avoid the loss of reducing properties.

Bottling and Storage. The bottles were manually filled with 740 mL of the indigo carmine solution. Then they were placed, one by one, under a head corking machine equipped with a gas flusher (N_2) and purchased from Pesce S.N.C (Asti, Italy); 10 mL of sodium dithionite solution was added to the bottle with the bottle-top dispenser, just before the closure was inserted. Due to the loss of reduction properties of the sodium dithionite solution, we found it suitable not to prepare more than 50−60 bottles using the same sodium dithionite solution (10−15 min); after this time had passed, the bottling process was stopped and a new sodium dithionite solution was prepared. To evaluate the variability among and within the lots of the natural cork stoppers and the synthetic closures, a total of 200 samples were bottled: 10 replicates of each of the 10 natural cork stoppers in the first trial and 10 replicates of each of the 10 synthetic closures in the second one. All of the bottles were labeled and stored vertically under a constant temperature of 26 ± 1 °C in a dark room over 3 months.

Colorimetric Measurement. A Minolta CR 400 spectrocolorimeter equipped with a DP 400 data processor (Osaka, Japan) was used to collect L^*, a^*, and b^* values (CIELAB76). To obtain the L^*, a^*, and b^* values, the bottle was inserted in an in-house-made steel tube with an inside diameter of 75 mm (slightly larger than the bottle body), a height of 160 mm, and a 32 mm diameter hole cut at the height of 90 mm from the bottom. The head of the spectrocolorimeter perfectly fitted the hole (**Figure 1**). The inside surface of the tube was subjected to a nickel plating process to optimize the performance of the spectrocolorimeter. Bottles of different shapes need specific tubes to avoid the entrance of light during the scanning. The chromatic measurements were collected using illuminant D65, and two measurements were done for each sample. Data were collected once a week, and oxygen diffusion rate (mg/month) was calculated as the slope of the line that interpolates the data related to the oxygen entry through each closure (mg) over time (day).

Calibration Procedure. The calibration curve was built by means of the same bottles and the same closures used for the sample preparation. A Kel-F Hub KF722S point style 5 needle (Hamilton, Bonaduz, Switzerland) fitted with a Teflon gastight (Hamilton, Bonaduz, Switzerland) valve was inserted through the closure. The valve had to be closed. One milliliter of air was injected into the bottle connecting a 1 mL Teflon gastight 1001 syringe (Hamilton) to the valve that was open only to permit the entry of the air through the closure (**Figure 1**). After the removal of the syringe, the bottle was shaken vigorously by hand for 2 min, to aid the dissolution of oxygen into the indigo carmine solution. The bottle was then inserted into the steel tube to collect the tristimulus values (L^*, a^*, b^*) by means of the spectrocolorimeter. The same procedure was repeated to add new controlled quantities of oxygen in the bottle until the obtainment of a dark blue color of the solution and constant tristimulus values. To optimize the results, the calibration procedure was performed on three bottles, and a new calibration curve was achieved for each bottling trial.

Stability of Indigo Carmine Solution. Eighteen Pyrex bottles were filled with 100 mL of indigo carmine solution and stored under different condition:

First Trial. Six samples were stored in a dark room at 20 °C (three in closed bottles to avoid direct oxygen contact and three in open bottles to permit an important oxygen dissolution in the solution).

Second Trial. Six samples were stored in a dark room at 40 °C (three in closed bottles and three in open bottles).

Third Trial. Six samples were store in sunlight at 20 °C (three in closed bottles and three in open bottles).

The absorbance at 610 nm and the absorption spectrum were recorded every 24 h for 7 days in 10 mm pathlight cuvettes, for all of the samples. Spectrophotometric analyses were carried out with a Jasco Corp. V-530 UV−vis spectrophotometer (Tokyo, Japan).

Data Analysis. All chromatic data were analyzed by means of Microsoft Excel XP software. Analysis of variance (ANOVA) was carried out with Statistica 8 (StatSoft Inc., Tulsa, OK). Absorption spectra were analyzed by using Jasco Corp. Spectra Manager for Windows 95/NT software (Tokyo, Japan).

RESULTS AND DISCUSSION

The aim of this investigation was to simplify and speed the procedure, proposed by previous studies (*20*, *21*), to quantify the amount of oxygen diffusion into bottles, with a nondestructive colorimetric method. The opportunity to analyze a large number of samples will allow an easier evaluation of the performance of different closures and of the variability within single lots.

Bottling and Colorimetric Measurement. The indigo carmine solution was completely saturated with oxygen during its preparation, to standardize the bottling condition, and just before the closure was inserted, an excess of sodium dithionite solution was added to the bottle to completely reduce the indigo carmine. Thus, it is not necessary to flush nitrogen in each sample to remove all of the oxygen present and to place the bottle in the corked machine under a continuous flush of nitrogen to avoid oxygen recontamination (*20*). As a result, it is possible to prepare

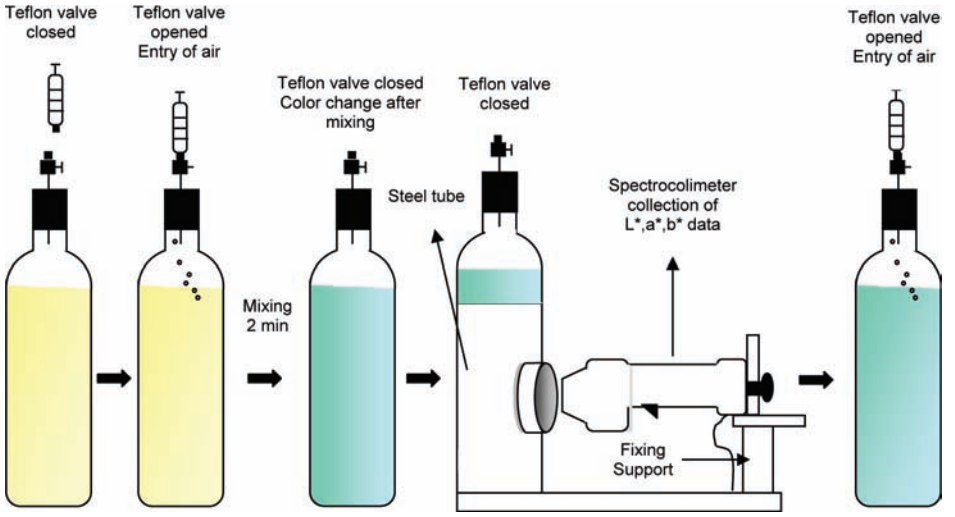

Figure 1. Diagram of calibration procedure and collection of chromatic measurements (L^*, a^*, b^*).

more or less 1000 samples a day, with relatively low-cost equipment.

The excess of sodium dithionite consumes all of the oxygen present in the indigo carmine solution and in the headspace of the bottle, and the color starts to change some days after the bottling. The study was conducted under the condition of a 15 g/L sodium dithionite solution, corresponding to 0.2 g/L in the bottles, which proved to be sufficient for our purposes. By adjusting the concentration of sodium dithionite, it is possible to advance or delay the beginning of the reaction.

The colorimetric scanning of each sample should be done in a dark room, as the presence of light does not permit the collection of reliable data. When the number of samples to analyze increases, the use of the steel tube, in which the bottle is inserted, makes the procedure simpler, allowing us to operate in a more manageable environment, and increases the accuracy of the measurements, standardizing the external conditions.

Calibration Curves. The reduced indigo carmine solution was reoxidized by injecting controlled oxygen volumes. Every oxygen addition led to a color change from yellow to dark blue that was subjected to colorimetric measurement. In previous studies the calibration curves were built by means of special bottles, dissimilar from those used for the samples. The addition of an excess of sodium dithionite and the adoption of a Teflon gastight valve allowed us to build the calibration curves using the same bottles and the same closures chosen for the sample preparation (**Figure 1**). Thus, the effect of bottle type on colorimetric measurement is eliminated and a specific calibration curve can be obtained for every commercial wine glass bottle we decide to test.

The calibration procedure should start some hours after the bottling to allow the adjustment of the closure to the bottleneck. The amount of oxygen added with each injection depends on the air temperature and pressure and is calculated according to the equation

$$\text{oxygen (mg)} = V \times D \times \%w \qquad (1)$$

where V is the volume of air added with each injection (mL), $\%w$ is the percentage of oxygen by weight in the air (0.232), and D is the density of moist air, which is calculated according to the equation

$$D = 1.2929 \times (273/T) \times [(B - 0.3783e)/760] \qquad (2)$$

where T is the absolute air temperature, B the barometric pressure (mmHg), and e the vapor pressure of the moisture in the air (mmHg). The factor $[(B - 0.3783e)/760]$ is obtained from a specific table (*23*) at different barometric pressures.

Previous studies (*20*−*22*) used an exponential relationship between L^* and oxygen content to perform a calibration curve that allows the estimation of the oxygen diffusion through the closures, as it permits the decrement of the error level due to the employment of different bottles in the calibration procedure.

In this study the calibration curve was obtained by adopting a cubic order polynomial relationship between the amount of injected oxygen and $1/\arctan(a^*/b^*) \times 100$. This type of correlation allowed us to reduce the asymptotic tendency of the L^* versus oxygen content relationship which, in our opinion, does not permit the collection of reliable data in the last section of the curve (**Figure 2**). An example of calibration curve is given in **Figure 2B**, where y is the $1/\arctan(a^*/b^*) \times 100$ value detected by colorimetric measurement and x is the quantity of oxygen migrating through the closure system.

Degradation of Indigo Carmine Solution. After more or less 3 months of data collection, a degradation of the indigo carmine solution (from dark blue to deep yellow) in the samples that show a very high oxygen permeation rate (> 1 mg/month) was observed (data not shown). The same behavior was not observed in samples with very low or medium (< 1 mg/month) oxygen permeation rate. We decided to investigate this phenomenon to understand its origin and its implications on the proposed method. Three possible causes of degradation were identified: oxygen, light, and heat (*24*−*29*). Three trials were planned, combining the causes of degradation to simulate real storage conditions, and, for each trial, high oxygen entry (open bottles) and low oxygen entry (closed bottles) were compared.

The analytical data over a storage period of 7 days indicated a significant difference ($p = 0.001$) in the absorbance at 610 nm among open and closed bottles for each trial (**Table 2**), and a corresponding visible change in the color was observed.

The degradation kinetics of the absorbance at 610 nm (**Figure 3**) showed that the combination of oxygen and light was the most important cause of instability, followed by the combination of oxygen and heat. In closed bottles the degradation was higher in the samples exposed to heat. These findings indicate that light and heat accelerate the reaction between indigo carmine and oxygen. We suppose that with a low amount of oxygen, a low temperature, and a dark environment, a color change from light yellow (reduced solution) to dark blue (oxidized solution) takes place. If the entry of oxygen is higher, the reaction

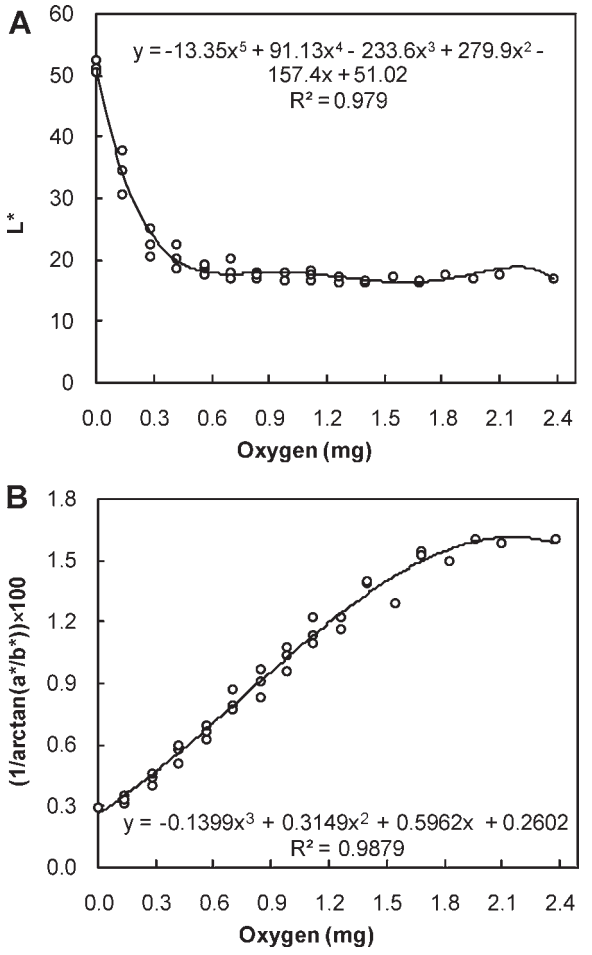

Figure 2. Calibration curves reporting L^* (**A**) and $(1/\arctan(a^*/b^*)) \times 100$ (**B**) parameters versus the amount of oxygen injected into the bottles ($n = 3$).

Table 2. Mean Absorbance (610 nm) of Open and Closed Bottles ($n = 3$) for Three Different Storage Conditions: First Trial (20 °C, Dark Room), Second Trial (40 °C Dark Room), Third Trial (20 °C, Sunlight)[a]

	absorbance 610 nm (au)		
trial	closed bottles	open bottles	difference between bottle types
first	3.372 (0.442)	0.356 (0.072)	***
second	1.848 (0.183)	0.110 (0.023)	***
third	2.460 (0.275)	0.011 (0.003)	***

[a] Parentheses enclose standard deviations. ***, significant at $p < 0.001$.

goes on to bring about the formation of another compound (deep yellow solution).

The spectral absorption (740−350 nm) was collected for all of the samples and for a previously reduced indigo carmine solution (addition of sodium dithionite) (**Figure 4**). Our data agree with the spectra reported in a previous study (*25*). The comparison between the reduced and degraded solution spectra seems to confirm that a high amount of oxygen, especially in presence of light or heat, leads to the formation of a new compound. Further research will be necessary to fully understand the reaction mechanism, the nature of the compound formed during the oxidative degradation, and the amount of oxygen that can be added to the solution before the beginning of the degradative reaction.

Due to these findings, we took some precautions to avoid errors in the interpretation of the data collected with the proposed

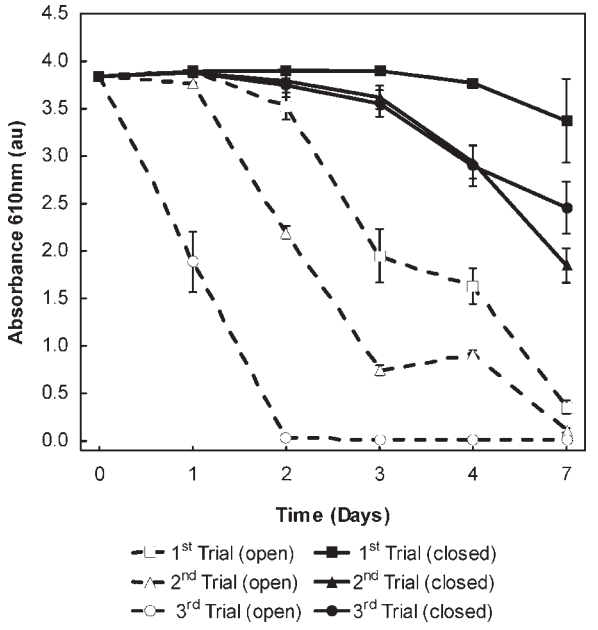

Figure 3. Degradation kinetic of the absorbance at 610 nm (au) for open and closed bottles ($n = 3$) at different storage conditions: first trial (20 °C, dark room), second trial (40 °C dark room), third trial (20 °C, sunlight).

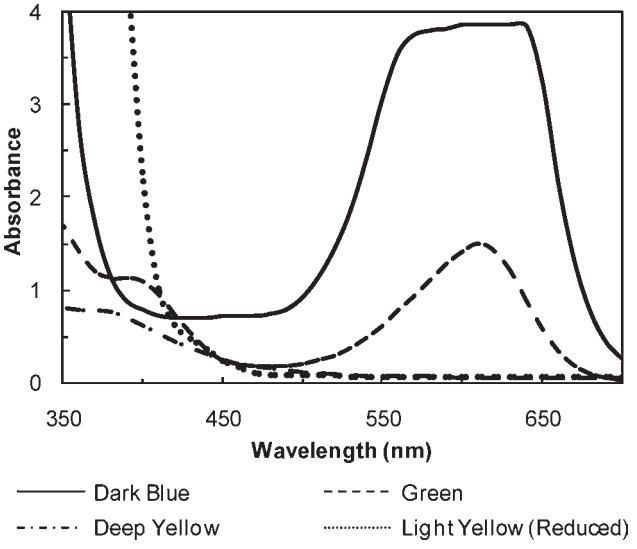

Figure 4. Spectral absorption (700−350 nm) of indigo carmine solution at different degradation levels (dark blue, green, deep yellow) and at the reduced state (light yellow).

colorimetric method. The samples were stored in a dark room at a controlled temperature of 26 ± 1 °C, and only the data collected during the first 3 months after the bottling were used to discuss the results.

Oxygen Diffusion Rate Variability among and within Lots. With regard to the natural cork stoppers, the change of color began 11 days after bottling. To better understand the behavior of the closures, we separately calculated the oxygen diffusion rate during the first month and during the second and third months after bottling. The analytical data collected by means of the spectrocolorimeter indicate significant differences ($p = 0.05$) in the oxygen diffusion rate among the closures in both of the periods we considered (**Table 3**), but whereas a high oxygen diffusion rate was observed during the first period, it drastically decreased and became steady at a very low value during the

Table 3. Mean Rate and Percent Coefficient of Variation (CV%) of Oxygen Diffusion through the Closures (n = 10)

closure	first-month (mg/month) means (CV%)[a]	second−third-month (mg/month) means (CV%)[a]	closure	first-month (mg/month) means (CV%)[a]	second−third-month (mg/month) mean (CV%)[a]
cork A	0.63 (34) ab[b]	0.12 (76) abc[b]	synthetic A	nd[c]	0.13 (23) a[b]
cork B	0.84 (49) b	0.09 (66) abc	synthetic B	nd	0.50 (12) ef
cork C	0.31 (92) a	0.02 (68) a	synthetic C	nd	0.31 (16) bc
cork D	0.47 (74) ab	0.13 (70) bc	synthetic D	nd	0.21 (38) ab
cork E	0.62 (72) ab	0.05 (97) ab	synthetic E	nd	0.38 (68) bcd
cork F	0.35 (25) a	0.10 (80) abc	synthetic F	nd	0.63 (17) f
cork G	0.31 (78) a	0.11 (102) abc	synthetic G	nd	0.46 (37) def
cork H	0.27 (65) a	0.07 (57) abc	synthetic H	nd	0.30 (23) abc
cork I	0.66 (52) ab	0.16 (37) c	synthetic I	nd	0.27 (15) ab
cork J	0.44 (77) ab	0.03 (41) ab	synthetic J	nd	0.36 (39) bcd

[a] CV% = (standard deviation/mean) × 100. [b] Different letters indicate significant differences ($p \leq 0.05$) among closures according to Tukey's HSD. [c] Not detectable.

second period. By comparing our data with those reported in previous studies (*20*−*22*) and converting them into the same unit, it is possible to verify similarity among the results. Our hypothesis is that the high oxygen diffusion rate observed during the first month after bottling could be due to the air forced out of the cork cells when natural cork stoppers are compressed into the bottleneck during the bottling, as already suggested in previous studies (*12*, *22*). We observed a great variability in oxygen permeation rate not only among the closures but also within single lots (**Figure 5**), and the phenomenon was particularly clear during the first month after bottling. Closures with high variability within the lots could lead, after a storage period, to wine bottles of extremely different quality. These results show that knowledge of the mean rate of oxygen diffusion is not enough to describe the behavior of a closure with regard to its oxygen permeation. A more complete description of the performances of a closure, and particularly with regard to the homogeneity of oxygen diffusion rate, is given by the coefficient of variation calculated on a large number of samples.

With regard to the synthetic closures, the change of color began 31 days after bottling. The synthetic closures we tested showed an oxygen diffusion rate lower than expected, and the amount of sodium dithionite added to the samples proved to be too high. Therefore, the delay we observed was ascribed to the excess of sodium dithionite. Due to this delay, we were not able to describe the kinetics of oxygen entry during the first month after bottling. The analytical data collected during the second and third months after bottling indicate significant differences (p = 0.05) between the closures (**Table 3**). Our data are partially dissimilar from those reported in previous studies (*20*−*22*), and generally we observed lower oxygen permeation rates. In those studies, only two kinds of synthetic closures were tested and, considering the heterogeneity of the products offered on the market, it is not odd if the results are different. Some closures exhibited a low oxygen diffusion rate (synthetic A and D), and others exhibited a high oxygen diffusion rate (synthetic F), so there appears to be a great variability in the behavior of different closure types. Except synthetic A and D, the oxygen diffusion rate of the synthetic closures is higher than those of the cork stoppers during the same period (second and third months), as shown in previous studies (*20*, *21*). With regard to the variability within the lots, we observed a great difference in the behavior of the tested synthetic closures (**Figure 5**): some of them exhibit very similar values for all of the replicates (synthetic A, C, and I); others showed a great variability in oxygen diffusion rate between the replicates. The behavior of the synthetic E was very

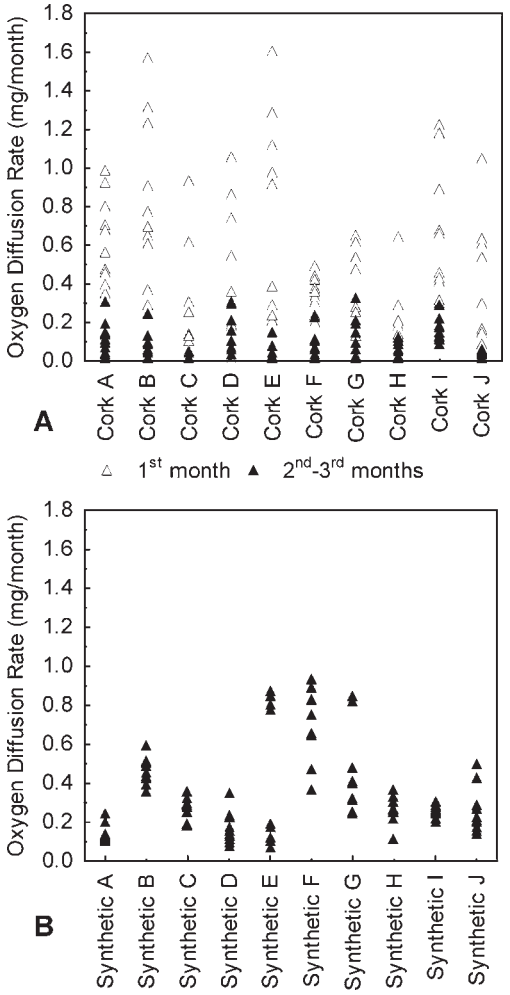

Figure 5. Variability of oxygen diffusion rate (mg/month) among and within lots of natural cork stoppers during the first and second−third months after bottling (**A**) and synthetic closures during the second−third month after bottling (**B**). All of the bottles were stored in a vertical position at a temperature of 26 ± 1 °C.

interesting: half of the replicates exhibit a high oxygen diffusion rate, whereas the other half exhibit a low oxygen diffusion rate. In such situations the mean value alone could be misleading, and the data regarding the variability of oxygen diffusion rate within the lot is essential to understanding the behavior of the closure and the risk of obtaining, after a storage period, wine bottles with different sensory properties.

Actually, the steadiness of the performance within single lots of stoppers, particularly with regard to oxygen permeability, could become a fundamental parameter in quality evaluation of closure systems (*30*) with the aim of warranting a homogeneous wine evolution during postbottling storage. Due to its natural origin, the performance of cork stoppers cannot be easily standardized. Instead, the field of synthetic closures could have a better opportunity for development, thanks to the possibility of controlling the manufacturing process to obtain a steady performance for single lots of closures.

Further research will be necessary to better understand the correlation between the variability of the oxygen diffusion rate of a single type of closure and the presence of different types of spoilage (e.g., oxidative aromas, color change) in only a limited number of bottles from a bottling run. Moreover, it will be necessary to elucidate the relationship between the oxygen diffusion rate of the closures and the sensory properties of the wines, to

find the right range of oxygen that every wine needs to express all of its potential during postbottling storage.

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