Free Sulfite in Beers—Kinetic Studies[1]

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ABSTRACT

The first part of the presentation is a theoretical approach to the different factors affecting the equilibrium between free and bound sulfite. It emphasizes the effect of dilution of the sample, concentration of reagents, time of reaction, and interfering substances that can alter the results of an experimental determination of free SO_2. The second part describes a micromethod for the direct determination of free sulfite in beers with hydrogen peroxide as an oxidizing agent. H_2O_2 instantaneously oxidizes the free sulfite, although it does not appreciably react with the usual components of the medium. Hydrogen peroxide is titrated according to a colorimetric method based on the release of methylene blue by oxidation of the leucodye in the presence of peroxidase. The sample volume is usually 50–100 μl. This method complies with the theoretical requirements. The third part is devoted to the applications of this method to the kinetics of fixation of SO_2 by beers and to the protection afforded by SO_2 as an antioxidant. Model solutions confirm that the oxidation of SO_2 by O_2 occurs chiefly through coupling with the oxidation of ascorbic acid. SO_2 largely prevents the peroxidatic phase, actually protecting the oxidizable polyphenols against oxidation. At least a fraction of bound SO_2 shares this property with free SO_2. A reliable microtitration method for bound SO_2 is urgently needed.

Key words: *Ascorbic acid, Beer, Coupled oxidation, Free sulfite, Microtitration*

In this article in line with our previous studies on the mechanisms of beer oxidation, we shall use the terms SO_2 and sulfite interchangeably to designate all ionized or nonionized forms of sulfur dioxide in solution. SO_2 is a minor natural component of fermentation products and results from the metabolic activity of yeasts, which are capable of reducing sulfates. In beers, the total amount is generally lower than 10 mg/L (20), although some yeast strains produce more SO_2 than others; in wines, the concentrations can substantially exceed 100 mg/L (23,24). As an additive, SO_2 has long been used to preserve a number of foods from the deleterious effects of molecular oxygen. Its role derives from several different mechanisms, which have been the subject of countless papers. As an oxidizable substance, SO_2 preferentially binds O_2, giving the harmless $SO_4^{=}$ ion. This is the commonly accepted view. As a reagent of the carbonyl function, it can block aldehydes and ketones, giving bisulfite addition compounds. This property is used for conserving the fresh bouquet of white wines. It is an effective inhibitor of several enzymatic reactions and delays the appearance of certain off-flavors and colors. It plays the role of an antiseptic toward numerous microorganisms. Only free SO_2 is effective in this last role.

The pH value largely affects the stability of the bisulfite addition compounds (4). The result depends on the reactivity of the carbonyl group and on the concentrations of the reactants. A strong basic pH value destroys all bisulfite compounds.

We shall limit this study to what happens in aqueous solutions in the normal pH range of beers (3.9–4.4) and especially at the pH 4.1, which is the pH of our special techniques. The pK_1 of SO_3H_2 is 1.77 (18); hence, at pH 4.1, 99.5% of the sulfite is in the form of SO_3H^-. The possibility of losses by volatilization is therefore almost completely excluded.

Our present knowledge of the reactions SO_2 leads to in a complex mixture derives from the application of analytical methods devised for the titration of SO_2 in a simple medium. These methods have several drawbacks. They are generally time-consuming and not sufficiently accurate for use as micromethods.

SO_2 is present in a complex medium in two forms, referred to as free and bound (or combined) sulfite. The free sulfite alone is oxidizable by iodine in acidic medium, but combined SO_2 is not. The speed at which the equilibrium between the two forms takes place is slow enough for such a distinction to be useful, at least in principle.

In fact, most of the classic methods are not applicable for titrating free SO_2 in beer or wine. The reactions are based upon the use of energetic oxidizing agents (iodine, permanganate, hydrogen peroxide), which also react in varying degree with several classes of organic compounds. Hence, an entrainment of free SO_2 is frequently used as a first step, SO_2 being trapped into a suitable oxidizing agent, like H_2O_2 at a fairly high concentration.

This first step produces an artifact that obviously disturbs the original equilibrium more as the conditions of the entrainment techniques differ from those prevailing in the original samples, namely pH value, temperature, and duration of treatment.

The classic iodine determination is not well adapted to solutions with SO_2 concentrations less than $10^{-3}N$ (32 mg of SO_2/L). The speed of reaction between iodine and sulfite ($SO_3^{=} \rightarrow SO_4^{=}$) decreases with a decrease in concentration, blurring the end point even with a pure aqueous solution. Side reactions that can disturb the stoichiometry have also been mentioned when the concentration of iodine is comparatively low (21).

The oxidation of SO_2 to SO_3 by H_2O_2 is a reaction both quantitative and rapid. In the classic procedures, it is frequently followed by an acidimetry of the sulfuric acid produced or by a complexometric titration of the excess of barium ions after the precipitation of sulfate is achieved. This method is far from having the sensitivity that would make it of interest for titrating the low concentrations usually encountered. The gravimetric titration of $SO_4^{=}$ as $BaSO_4$ is time-consuming and not sufficiently accurate in the range of small concentrations. Nephelometric techniques have been proposed. One cannot always take advantage of their high sensitivity. Nevertheless, they can be used under definite conditions for determining small amounts of sulfate, even in the presence of sulfite (11).

The direct colorimetric method of Stone and Laschiver (19) (using *p*-rosaniline chloride decolorized in strong acidic medium) has an excellent sensitivity (mg/L range). It requires numerous pipettings and is accordingly hardly useful as a rapid micromethod. The reduction of total SO_2 to H_2S with $SnCl_2$, followed by a colorimetric determination of the methylene blue (MB) formed in a subsequent step has been proposed (2) but is not suitable for the determination of free sulfite. The use of the sulfite selective electrode developed by Orion Research Inc. (Cambridge, MA; model 95.64) seemed to be promising because of the possibility of continuous recording common to this type of technique. Still, it requires the acidification of the sample to pH 1.3, which does not suit our requirements.

EQUILIBRIUM BETWEEN FREE AND BOUND SULFITE

For a good understanding of the part played by the various factors that govern the relationships between free and bound SO_2 in a complex medium, it is necessary to consider the simplest cases and to illustrate the results graphically.

Let us consider first the general case in which only one carbonyl compound, for example an aldehyde A, is mixed with sulfite. An

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equilibrium ensues that is governed by the law of mass action:

$$A{\cdot}SO_2 \rightleftharpoons A + SO_2$$

Assuming that a = initial concentration of the aldehyde, t = total concentration of sulfite, f = concentration of the *free* sulfite, c = concentration of *combined* (bound) sulfite (c = t − f) (which is also the concentration of the bisulfite addition compound $A{\cdot}SO_2$), K = the equilibrium constant of the reaction at a given temperature and pH, and r = the ratio a/t, the constant K is given by

$$K = \frac{(a - t + f)\,f}{t - f} \qquad (1)$$

which can also be expressed by

$$f^2 + [K + (r - 1)\,t]\,f - Kt = 0 \qquad (2)$$

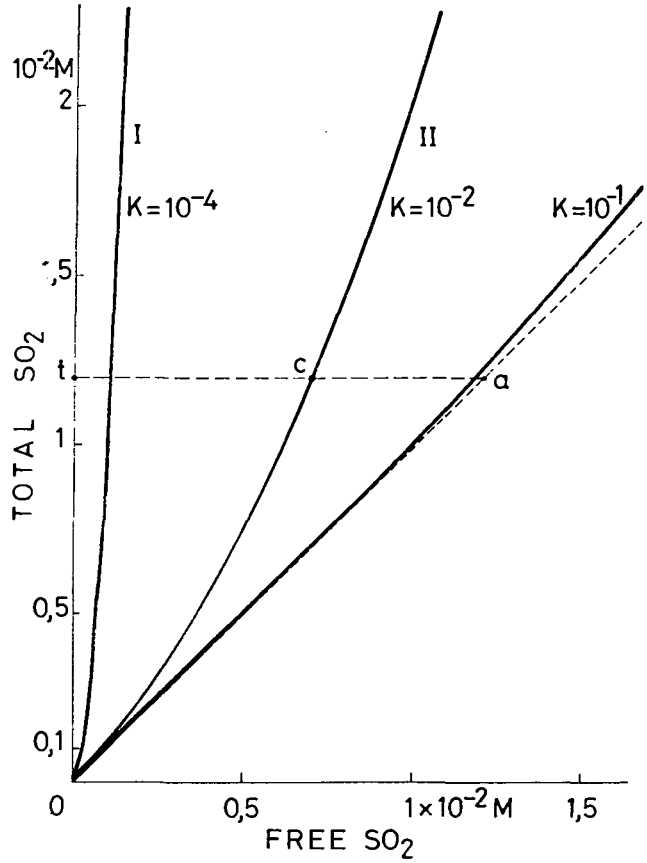

Fig. 1. Effect of a change in concentration on mixtures of aldehydes + SO_2. The value of the constant K and the aldehyde/SO_2 ratio determine the shape of the parabola. All of them have the tangent +1 at the origin. I = curve for small K, II = curve for medium K, at = total concentration, tc = concentration of free SO_2, ca = concentration of combined SO_2.

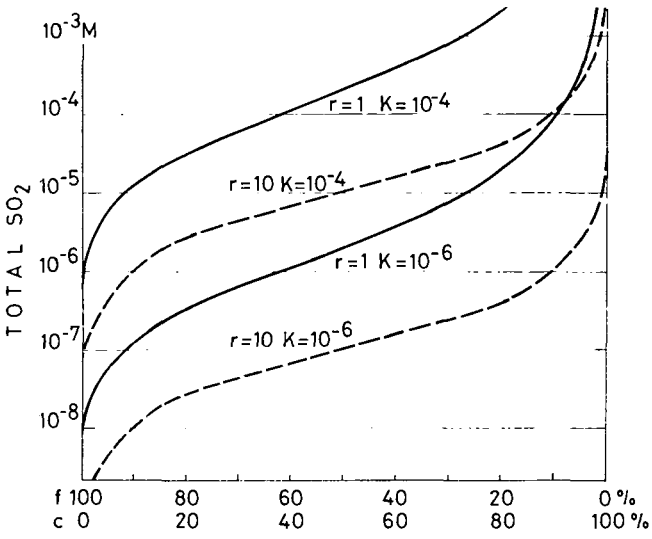

Fig. 2. Effect of dilution on the composition of two SO_2/aldehyde compounds of different stability. ——— = aldehyde concentration = SO_2 concentration, ... = aldehyde concentration 10 times greater than SO_2 concentration. Reference is made to the concentration of SO_2. f = free sulfite, c = combined sulfite, r = ratio, K = constant.

The mathematical treatment leads to the following results: The curves are parabolas more or less "open" according to the values of K and r. For each of them, the useful part is limited to the portions given as an example in Fig. 1 for three arbitrary values of K. All the curves have the same tangent value, + 1, at the origin.

If K is small (high affinity of SO_2 for A) the curve is of type I. If K is medium (moderate affinity of SO_2 for A) the curves are of type II. A horizontal line drawn for a definite concentration (t = ot = ta) gives the concentrations of f (= tc) and c (= ca).

One sees immediately that, for *increasing dilutions* of a given bisulfite addition compound, the percentage of free SO_2 increases; it tends to be equal to the total SO_2 for very great dilutions. The result of dilution is still more striking when seen as in Fig. 2, which gives the free SO_2 as a percentage of total SO_2.

The composition of the mixture is plotted versus the concentration of total SO_2, taking for K two arbitrary values, eg, 10^{-6} and 10^{-4}. They can obviously be deduced from one another by translation (Table I).

If we consider r values other than 1, the shape of the curves is altered. For example if r = 10, one gets the dotted curves corresponding to the two preceding ones. A dilution in a given range affects the percentage of free sulfite very differently depending on the values of the constant K and the ratio of aldehyde to sulfite.

Let us study now, how the concentration of the combined sulfite varies when *increasing amounts of* SO_2 are added to a dilute

TABLE I
Variation in Percentage of Free SO_2 as a Function of Total SO_2 for Two Bisulfite Carbonyl Compounds of Different Stability Constant K and for Two Molar Ratios[a]

	Molar Ratio			
	1		10	
	K Value		K Value	
Total SO_2	10^{-6}	10^{-4}	10^{-6}	10^{-4}
2×10^{-9}	···	···	98.0	···
10^{-8}	99.0	···	91.0	···
2×10^{-8}	98.1	···	83.5	···
5×10^{-8}	95.4	···	67.4	···
10^{-7}	91.5	···	51.2	···
2×10^{-7}	85.4	···	34.2	98.0
5×10^{-7}	73.2	99.5	17.8	95.2
10^{-6}	61.8	99.0	9.9	91.0
2×10^{-6}	50.0	98.1	5.23	83.5
5×10^{-6}	35.8	95.4	2.17	67.4
10^{-5}	27.0	91.5	1.09	51.2
2×10^{-5}	20.0	85.4	0.55	34.8
5×10^{-5}	13.2	73.2	···	17.8
10^{-4}	9.51	61.8	···	9.9
2×10^{-4}	6.82	50.0	···	5.23
5×10^{-4}	4.37	35.8	···	2.17
10^{-3}	3.11	27.0	···	1.09
2×10^{-3}	2.21	20.0	···	0.55

[a] Molar ratio = initial concentration of aldehyde/total concentraton of sulfite.

solution of carbonyl compounds. We take as an example *acetaldehyde* and *pyruvic acid* (buffered at pH 4) where the stability constants K are in a ratio close to 100, 2.7×10^{-6} and 3×10^{-4}, respectively (1), at a concentration of $10^{-3}M$ (44 and 88 mg/L).

We write the law of mass action, taking for concentrations the following symbols: T = total carbonyl compound, F = free form, C = combined form. (C is obviously equal to c). We obtain:

$$c^2 - (t + T + K)c + tT = 0 \qquad (3)$$

Different values of c were calculated for total SO_2 values in the range $0–3 \times 10^{-3}M$ (0–192 mg/L) and plotted in Fig. 3. With acetaldehyde, most of the SO_2 is combined over almost the whole range: 98% of the SO_2 is still bound for a concentration of $0.87 \times 10^{-3}M$, whereas with pyruvic acid, only 80% is bound for an SO_2 concentration as low as $10^{-5}M$.

For a mixture of the two carbonyl compounds, each at the concentration of $10^{-3}M$, the curve is, in fact, the succession of the two preceding ones. Acetaldehyde binds practically all the SO_2 added until its concentration reaches $10^{-3}M$. Then pyruvic acid binds SO_2 as if it were alone (Fig. 4, solid curve). The dotted curve in Fig. 4 clearly shows that the percentage of the free sulfite increases steadily as total SO_2 is increased, but its concentration depends on the nature and concentration of the different carbonyl compounds, of which the affinity for SO_2 is accounted for by the constants K of their bisulfite addition compounds.

In a mixture of various carbonyl compounds of which the composition is not completely known, the free sulfite content cannot be deduced from the added SO_2 amount. This is consistent with the results obtained by different authors with wines (1) and ciders (4).

SEARCH FOR A METHOD TO DETERMINE FREE SULFITE

Aim of the Study

Our aim was to improve our knowledge of the oxidation mechanism of SO_2 by O_2 in solutions of increasing complexity in the presence of various catalysts to accurately define the part SO_2 can play as an antioxidant and its limits. The distinction between free and bound SO_2 imposes itself from the very beginning according to the differences in their reactivity. We needed a method for the determination of free SO_2 that was simple, quick, sensitive, hardly affected by interferences of organic compounds usually present in the medium to be studied, and that complied with the requirements seen in the foregoing section. The study started with pure aqueous solutions of sulfite of concentrations less than $10^{-3}M$ and then was extended to binary mixtures: SO_2 + ascorbic acid (AH_2), SO_2 + carbonyl compounds, SO_2 + catechin, ternary mixtures $SO_2 + AH_2$ + catechin and $SO_2 + AH_2$ + aldehydes, and finally to beers.

Iron Dipyridyl Complex Method

Our previous work has shown what kind of information can be obtained from the application of the iron dipyridyl complex ($DPFe_3$) method to beers (8). The considerable difference in the oxidation speed of various oxidizable substrates as well as the limits attained can be used in the selective microtitration of components of definite mixtures, for example, in the study of the oxidation of a mixture of AH_2 + catechin. We tried to apply this method to mixtures of $AH_2 + SO_2$, which led us to go into details of the formation of the red dipyridyl ferrous complex (DPFeII) on reduction of $DPFe_3$ by SO_2 and to rectify an error we overlooked in our previous communication (8). According to the curve drawn for SO_2 (Fig. 2, p. 309 of that paper) the oxidation of $SO_3^=$ to $SO_4^=$ appears to require two equivalents, which could reasonably be expected but is far from being true.

When sulfite is added to $DPFe_3$ in the cuvette of the colorimeter, the transmittance decreases progressively and reaches a plateau after 10 min. If the absorbances are plotted against the amount of added SO_2, a curve is obtained (Fig. 5), not a straight line as in the

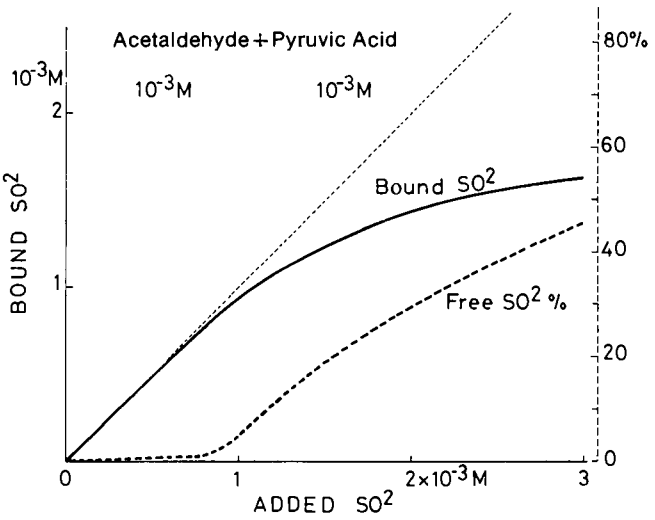

Fig. 4. Effect of increasing SO_2 concentration on the percentage of free sulfite for an equimolar mixture of acetaldehyde and pyruvic acid.

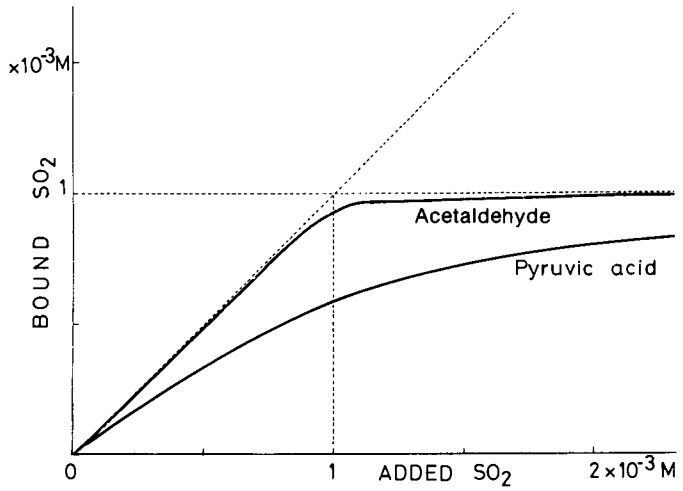

Fig. 3. Effect of increasing SO_2 concentration on the free/bound SO_2 equilibrium for two carbonylic compounds with different affinities. Acetaldehyde/SO_2 $K = 2.7 \times 10^{-6}$; pyruvic acid/SO_2 $K = 3 \times 10^{-4}$.

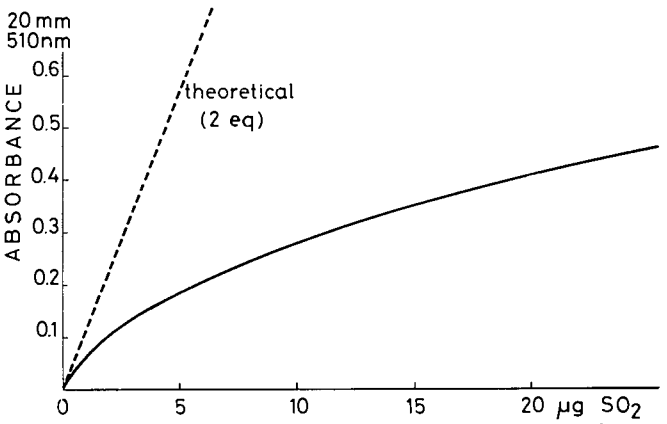

Fig. 5. Formation of the red divalent iron/dipyridyl complex (DPFeII) on reduction by SO_2 of the colorless trivalent complex (DPFe3) sharply decreases when the concentration of SO_2 increases. The absorbance values plotted in this diagram are read 10 min after the addition of the sample into the DPFe3 reagent after a stable reading has been obtained. The volume of the DPFe3 reagent is 4 ml.

cases of AH_2 or Fe^{++}. The yield of DPFeII, expressed as equivalents, decreases very rapidly with the amount of SO_2 added (Fig. 6).

With mixtures of SO_2 and AH_2, the oxidation shows two steps. AH_2 is instantaneously oxidized and leads to the expected increase of absorbance. However, the higher the amount of AH_2 added, the lesser the subsequent increase of absorbance due to the oxidation of the same amount of SO_2 (Δ O.D.). Nevertheless, Fig. 5 could actually be used for determining the dose of SO_2 brought in by the sample on plotting Δ O.D. due to the sulfite from the absorbance value obtained after oxidation of AH_2. However, as the curve goes down, the method loses a great deal of its sensitivity, and therefore our interest in it for the determination of small amounts of sulfite decreases.

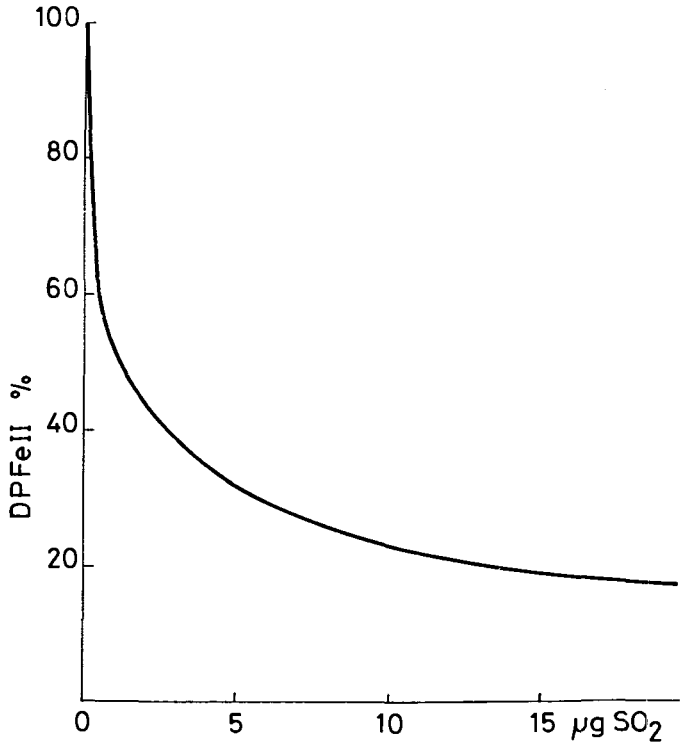

Fig. 6. Yield of dipyridylferrous complex (**DPFeII**) formation (in Fig. 5), given here as percentage of the theoretical value of two equivalents of SO_2 per mole.

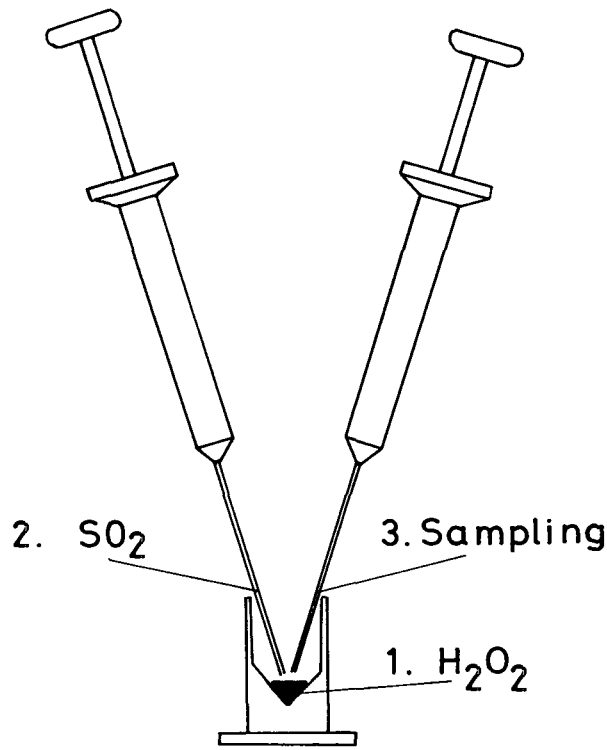

Fig. 7. Experimental scheme illustrating the principle of the microdetermination of free SO_2. Numbers indicate the order of the steps.

Bear in mind the very interesting possibility of excluding any interference of SO_2 in the titration of reducing substances like AH_2 or polyphenols. One can add 50 µl of H_2O_2, about 0.02*N*, at the beginning to $DPFe_3$ in the cuvette of the colorimeter. Under this condition, SO_2 brought in by the sample is instantaneously oxidized to SO_3 by H_2O_2 and does not give any DPFeII. On the other hand, the H_2O_2 present does not interfere in the oxidation of the classic reducing agents of $DPFe_3$; their determination remains unaffected.

MB Method

This method, whose possibilities were recently used for a microtitration of H_2O_2 (6), provides an excellent tool for a back titration of free sulfite and meets all requirements listed at the beginning of this section.

The base solution S (AH_2 + leucomethylene blue) to which peroxidase and reduced glutathione (GSH) have been added (the last compound for getting rid of an eventual contamination by copper ions), releases an amount of MB proportional to the amount of H_2O_2 added. This solution is largely insensitive to the presence of many other reducing substances like AH_2, polyphenols, and GSH at concentrations usually present in the sample.

Furthermore, experience shows that most of them react only very slowly with diluted H_2O_2 if no peroxidatic catalyst is present.

The principle of the determination is illustrated in Fig. 7. One pipets into a small polystyrene vessel a volume of H_2O_2 of known concentration such that after addition of the SO_2-containing sample, only a small excess of H_2O_2 remains. One immediately pipets an aliquot of this mixture into 5 ml of base solution S, which has been equilibrated by air bubbling in the colorimeter.

From the height of the peak due to the released MB (Fig. 8), one calculates, as formerly described, the amount of H_2O_2 brought in by the sample, hence, by difference, the SO_2 equivalent to H_2O_2 that has reacted, taking into account the amount of H_2O_2 added and the dilutions used.

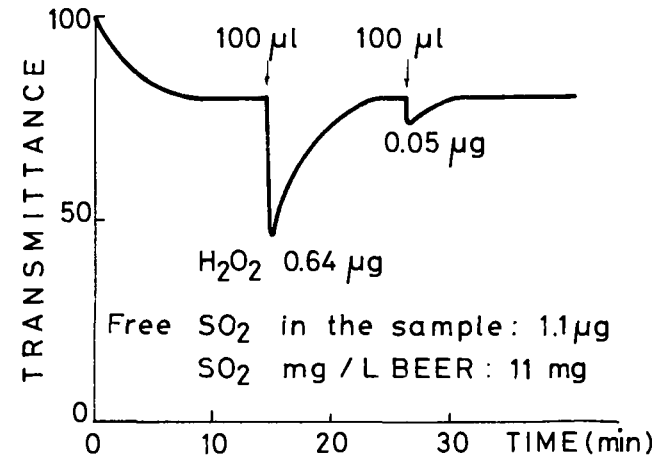

Fig. 8. Example illustrating the sensitivity range of the methylene blue (MB)-micromethod for the determination of H_2O_2. In the presence of peroxidase, H_2O_2 preferentially reacts with leucomethylene blue of the base solution S, rapidly releasing MB, which is in turn slowly reduced by the excess of ascorbic acid. The increase of MB concentration linearly varies with the amount of H_2O_2 given. Free SO_2 instantaneously reacts with H_2O_2, thus providing the possibility of a back titration.

The overall process does not last more than 10 sec. The reaction of H_2O_2 on the mixture is stopped as soon as an aliquot of this mixture is put into the base solution S, and the peak is reached in about 30 sec.

The relative accuracy is often greater than 5% when the volumes of H_2O_2 and SO_2 are suitably chosen and when the sample does not contain an oxidatic activity[2] insensitive to GSH. In the last case (iron complexes, oxidatic organic catalysts), it is still possible to make a determination of the excess of H_2O_2 by working with a slight bubbling of pure nitrogen instead of air.

KINETICS OF THE COMBINING OF SULFITE BY BEER

Before studying the oxidation of sulfite dissolved in beer, it is necessary to know more about the kinetics of binding. The reactivity of all beer samples toward sulfite is basically the same. Hence, description of some typical behaviors is justified.

Beer

Known amounts of SO_2 (for example 20, 50, 100 mg/L) are added to a beer sample and the free sulfite is determined on aliquots at intervals. Figure 9 illustrates the results obtained during the first hour. The binding is especially rapid. According to the theoretical development, the higher the amount added, the more SO_2 is bound. When the results are expressed in terms of percentage, this percentage clearly decreases with increasing amounts of sulfite added (Fig. 10).

Extending the study over a longer period (24 hr) does not significantly decrease the amount of free SO_2. However, for such long trials it is preferable to exclude air in order to prevent any interference of oxidation.

Elimination of free SO_2, from adding the calculated amount of H_2O_2, would be expected to be followed by the rapid settling of a new equilibrium. If a certain amount of sulfite is indeed released, this phenomenon is slow and of small degree. This observation leads us to think that only labile bisulfite addition compounds are concerned in this process.

For a "titration" of a "free SO_2 fraction" to be possible, it is, of course, necessary that the bound sulfite react only sluggishly with the oxidizing agent. Obviously, the result will depend in large measure on the *technique*, the *nature* of the reagent, its *concentration*, and the *reaction* time.

[2]As described previously (6) "oxidatic" and "peroxidatic" are used here to refer to reactions that have similarities to those mediated by the enzymes oxidase and peroxidase but that are not enzymatic in nature; for example, copper ions show "oxidatic" activity with ascorbic acid and iron ions show "peroxidatic" activity.

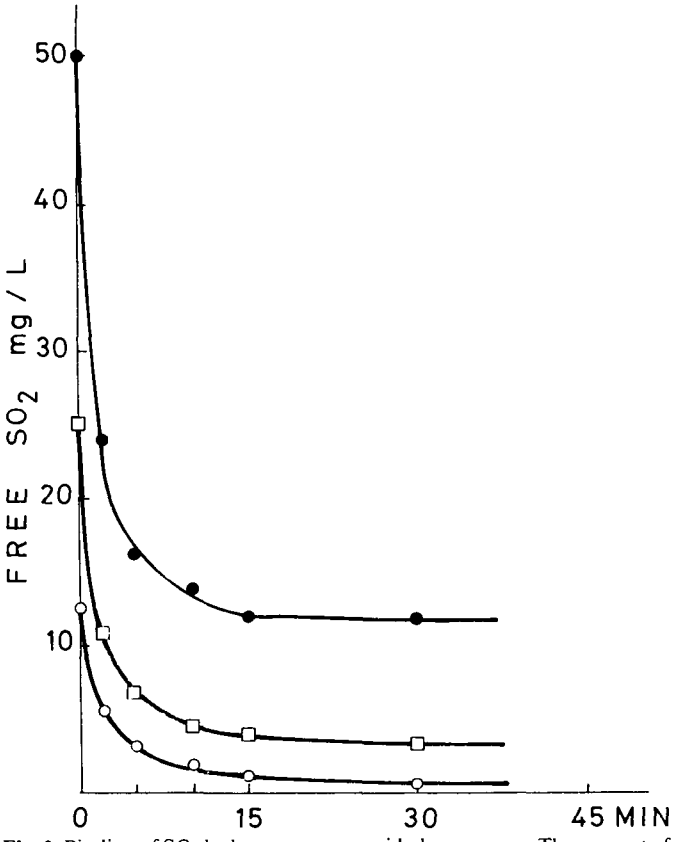

Fig. 9. Binding of SO_2 by beer as a very rapid phenomenon. The amount of bound SO_2 increases with increasing added sulfite. • = 50 mg/L, □ = 25 mg/L, o = 12 mg/L.

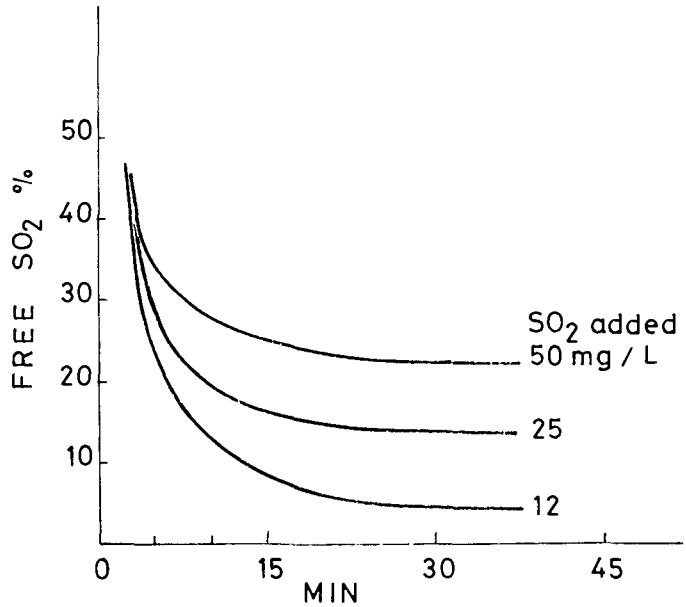

Fig. 10. Expressed as a percentage, the free SO_2 decreases as total SO_2 increases. The three curves correspond to those given in Fig. 9.

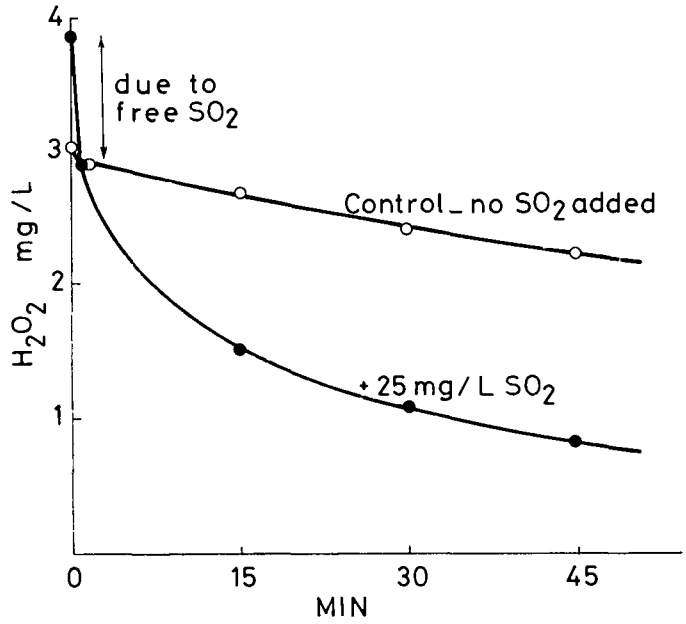

Fig. 11. H_2O_2 sluggishly reacts with the usual components of beers (control). But if the beer has been previously treated with SO_2, after the free SO_2 is oxidized (instantaneous reaction), some labile bisulfite addition compound slowly reacts with H_2O_2 (lower curve).

All this can be clearly demonstrated by the following two series of trials.

First Series. One adds 3 mg/L of H_2O_2 to a beer and follows its disappearance with time. The results are given in Fig. 11 (upper curve). In a second trial, one adds a definite amount of sulfite (eg, 50 mg/L) to the same beer. After a few hours, the free sulfite is determined. H_2O_2 is then added in an amount such that after the oxidation of free sulfite the excess of H_2O_2 is the same as in the first trial. The disappearance of H_2O_2 is followed over time, with the results given in Fig. 11 (lower curve). The difference between the two curves represents the oxidation of the labile bisulfite addition compounds that release SO_2 during the first minutes in the presence of a small concentration of H_2O_2.

The reaction is far from affecting all the bisulfite compounds.

Second Series. One adds a known amount of sulfite to a solution of propanal (5 µl/5 ml of water). One determines the disappearance of free sulfite by three methods: MB, $DPFe_3$, and p. rosaniline.

The results are illustrated in Fig. 12.

It is not surprising that the amount of bound sulfite appears to be *greater* with the MB method than with the $DPFe_3$ method. In the latter case, the oxidizing agent is used in large excess and the pH is different (2.6); the time necessary for complete oxidation of SO_2 in pure solution is 10 min.

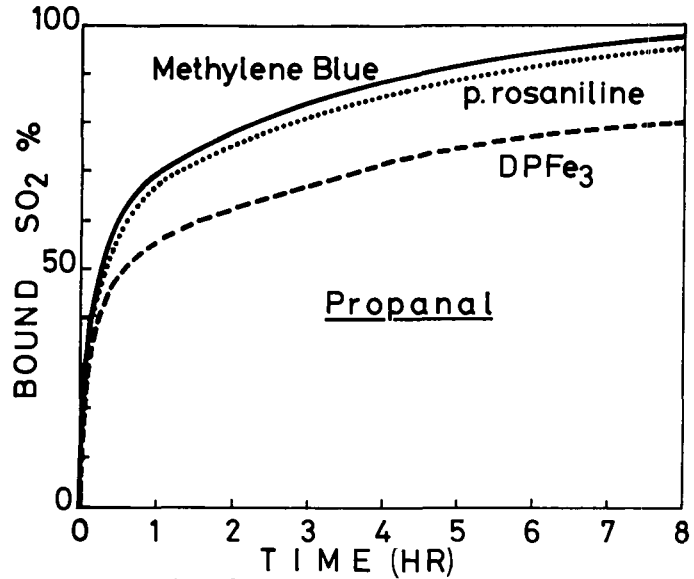

Fig. 12. The fraction referred to as "free SO_2" depends on the analytical technique used. Even for stable bisulfite addition compounds (SO_2/propanal), the results are higher with methods using high dilutions, strong reagents, and long duration. **DPFe3** = iron dipyridyl complex method.

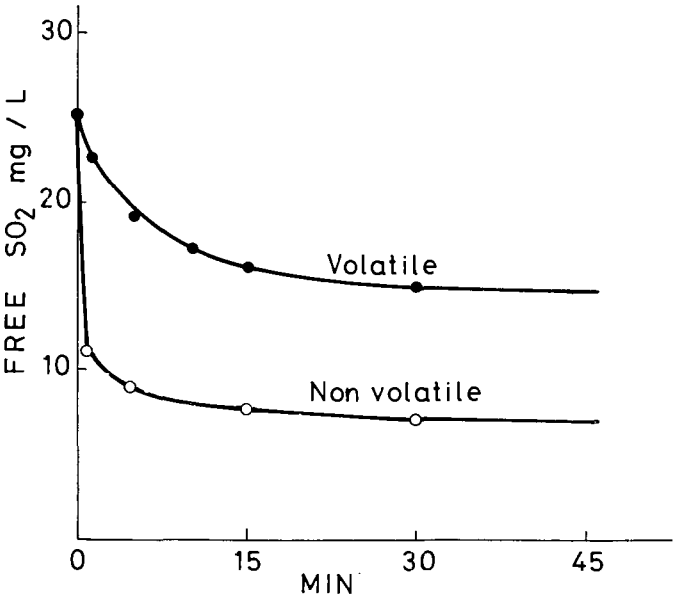

Fig. 13. The nonvolatile fraction of a beer displays a higher affinity for SO_2.

Combining Power of Different Fractions

Within the framework of studies on wines and ciders, countless attempts were made to define the role of the numerous carbonyl compounds in the binding of SO_2 in order to establish a balance sheet between free and bound SO_2. The time allotted for the equilibrium between beverage and sulfite to be achieved was generally longer than two days. In some few instances, it was actually possible to calculate the effective free SO_2 from the total amount added, the concentration of the main carbonyl compounds, and the constant K of their respective bisulfite compounds. In most cases, there were huge discrepancies; up to 30% of the bound sulfite could not be accounted for (1,4).

To our knowledge, no similar study has been made so far with beers, and it is not our aim to tackle such a work. However, it is useful to get a general view of the combining power of different easily attainable fractions, such as the volatile, nonvolatile, and nondialyzable fractions.

In a first trial, the beer was distilled until about 50% of the original volume was recovered. Distillate and residue were brought to the initial volume with water.

In a second trial, the beer was dialyzed against distilled water during 24 hr in a preweighed bag of collodion. After dialysis, the bag was suspended in air until it recovered its original weight. The original beer and the three fractions were treated with the same amount of sulfite, and the combining of SO_2 was followed over a period of time, as described above. The results are illustrated in Fig. 13 and qualitatively confirm the work of Stone and Laschiver (19). The residue or nonvolatile fraction has the highest combining power, followed by the volatile fraction. The macromolecules retained in the dialysis bag (proteins and carbohydrates) had negligible combining power.

If, *before* addition of sulfite, a beer is treated with small

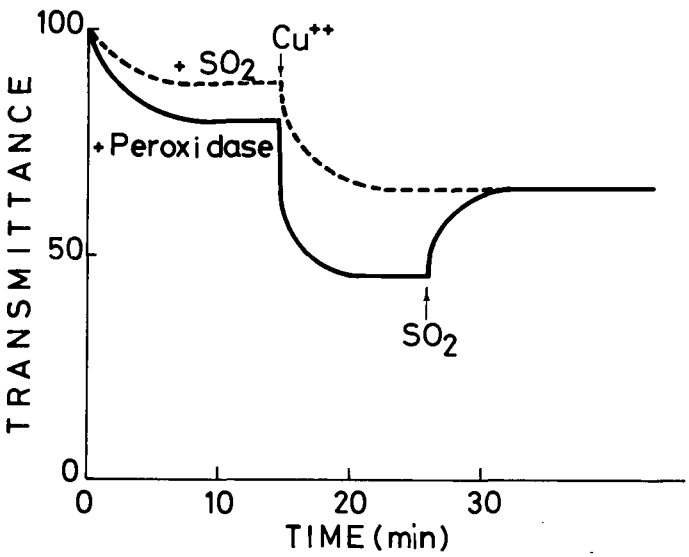

Fig. 14. The colorimetric methylene blue (MB) method immediately shows that in the presence of SO_2 (------) the equilibrium concentration of MB is reduced to approximately 50% of that in the presence of peroxidase. (———). Because SO_2 instantaneously binds H_2O_2 formed in the oxidatic step, it precludes the peroxidasic step and hence decreases the concentration of MB by 50% (——— after the addition of SO_2).

concentrations of the classic reagents known to react with the carbonyl function (hydroxylamine, hydrazine, phenylhydrazine, and dinitrophenylhydrazine, which do not interfere with the determination of H_2O_2 in the MB methods), the binding of sulfite is considerably reduced but not completely suppressed. The addition of the same reagents *after* the sulfite does not release a significant amount of sulfite.

OXIDATION OF SO_2 IN THE PRESENCE OF ASCORBIC ACID

Binary Mixture $SO_2 + AH_2$

An aqueous solution containing 10–100 mg of SO_2 per liter at about pH 4 oxidizes itself very slowly, even in the presence of copper or iron ions at a concentration of a few milligrams per liter. *If AH_2 is added, the oxidation of SO_2 follows the oxidation of* AH_2, which imposes its own speed. This can be easily shown by both the MB colorimetric and manometric methods.

Colorimetric Method. If a small amount of SO_2 (eg, 50 μl of a 0.05*N* solution) is added to the base solution S in the cuvette of the colorimeter, the equilibrium concentration of MB for any amount of any oxidatic catalyst is reduced to *about* half of that reached in the presence of peroxidase (Fig. 14). The colorimetric method clearly illustrates that while scavenging H_2O_2, SO_2 inhibits the production of MB in the peroxidatic step. The concentration of MB obtained decreases somewhat when the dose of SO_2 is further increased.

Accordingly, it can be expected that SO_2 is able to suppress every oxidation phenomenon bound to the peroxidatic step, as has been shown (8). This accounts for most of its protective effect on the oxidation of polyphenols, for example. However, this colorimetric technique gives information only on the initial step of the reaction. The manometric method, on the contrary, enables the analyst to follow the O_2 consumption until complete oxidation of AH_2 is achieved.

Manometric Method. Using a manometric measure of the consumed O_2 with continuous recording on 10 ml of a mixture of AH_2 ($4\times10^{-3}M$) + SO_2 ($10^{-3} - 1.2\times10^{-4}M$) buffered at pH 4, the periodic titration of free sulfite (MB method as described above), the determination of remaining AH_2 (DPFe$_3$ in the presence of H_2O_2), the estimation of formed sulfate (as SO_4Ba) according to a nephelometric procedure in the presence of gelatin (11) on sample volumes in the 5–50-μl range, we can draw the curves given in Fig. 15 and make the balance sheet of the oxidation during the whole course of the experiment.

One can immediately see that SO_2 leads to a noticeable decrease of the speed of O_2 consumption. Toward the end, the rate of O_2 consumption becomes so low that during several days, AH_2, SO_2, and O_2 coexist *in the solution* (especially with iron ions as a catalyst). This agrees with the results presented for beers by Brenner et al (3) and Van Gheluwe et al (22).

The kinetics are similar to the theoretical cases described in 1979 (5) when the redox potential of the catalyst lies below that of oxidizable compound.

According to the scheme proposed in our previous communication (6), SO_2 should only intervene in the peroxidatic step, without interfering with the oxidatic one. So far, we have no satisfactory explanation for the decrease of the speed of oxygen consumption, which occurs whatever the nature of the catalyst.

Ternary Mixtures

SO_2 + AH_2 + Polyphenol. This study was made on the solution defined in the preceding section, to which was added either catechin, or procyanidin B$_3$, or a trimer (the last two prepared according to Eastmond and Gardner [12,13]) so that the concentration of the phenolics was approximately 1 g/L. The presence of the polyphenol did not seem to alter the speed nor the course of the oxidation of the binary mixture. However, in the case of the trimer, in spite of the presence of SO_2, some oxidative polymerization occurred, as can be shown by thin-layer chromatography and by cinchonin sulfate (increasing of tanning power) (7). Therefore the protection against oxidation does not seem to be complete.

SO_2 + AH_2 + Propanal. Propanal (0.04*M*, 3.75 ml) and sulfite (0.04*M*, 0.75–5 ml) were mixed first in the main compartment of a Warburg flask. After 1 hr, AH_2 was added and the mixture brought to 9.5 ml with water. The catalyst (copper ions for a final concentration of 60 μg/L) was put into the side compartment. After shaking for 15 min in the water bath (25°C) for temperature equilibration, the catalyst (0.5 ml) was added (time zero).

This solution is an interesting model for understanding what happens in a beer treated by the classic antioxidant mixture AH_2 + SO_2. From the very beginning, a fraction of SO_2 binds itself with the aldehydes so that the concentration of free sulfite rapidly becomes a small fraction of the total SO_2.

Since the reactivity of H_2O_2 on combined sulfite is low, one can ask the question whether the oxidation of SO_2 by O_2 coupled to that of AH_2 is limited to the free fraction or whether the bisulfite

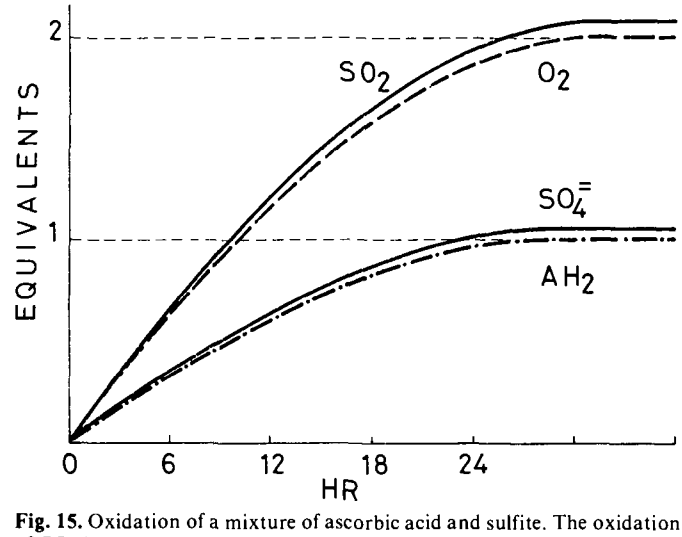

Fig. 15. Oxidation of a mixture of ascorbic acid and sulfite. The oxidation of SO_2 is coupled to that of ascorbic acid. Free SO_2 disappears twice as quickly because as soon as it is formed, dehydroascorbic acid gives a very stable bisulfite addition compound.

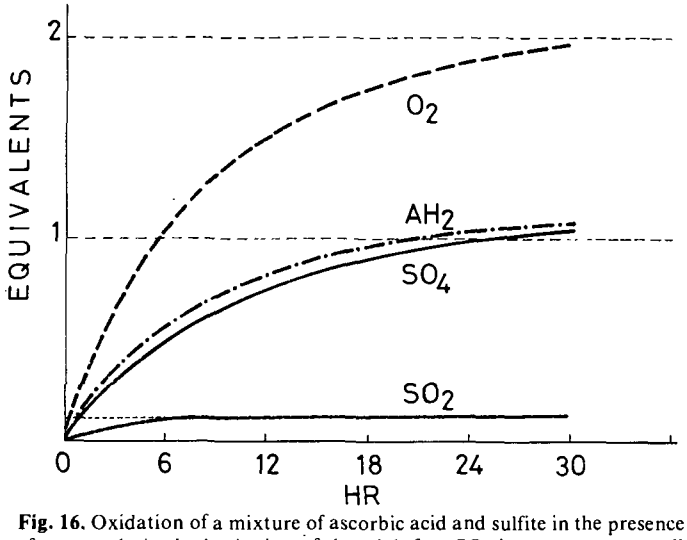

Fig. 16. Oxidation of a mixture of ascorbic acid and sulfite in the presence of propanal. At the beginning of the trial, free SO_2 is present at a small concentration (lower dotted line). It disappears completely after 6 hr. The concentration of $SO_4^=$ ions continues to increase, indicating that SO_2 bound to propanal supports a coupled oxidation.

addition compound, releasing all or only a part of its sulfite, participates in the formation of sulfate. The results are illustrated in Fig. 16, with a molar ratio of $SO_2/AH_2 = 3$.

It is clear that the formation of sulfate accurately follows the oxidation of AH_2. At the beginning, two equivalents of free sulfite disappear for each equivalent of oxidized AH_2, as is the case for the mixture $AH_2 + SO_2$. After a few hours, when free SO_2 is no longer present, the concentration of sulfate continues to increase, still following the oxidation of AH_2. Clearly the propanal bisulfite addition compound can play a part similar to that of free SO_2, at least in slow coupled oxidations.

OXIDATION OF SULFITE IN A COMPLEX MEDIUM

$SO_2 + AH_2$ + Beer

Because some carbonyl compounds of beer combine with SO_2, this trial directly derives from the preceding one. The speed of oxidation is still comparatively high and the study of this mixture can be made with the same methods.

Although $DPFe_3$ oxidizes both AH_2 and the usual reducing substances of beers (polyphenols and melanoidins), the trials showed that the decrease of the reducing power on oxidation was, in fact, only accounted for by the disappearance of ascorbic acid. This trial did not tell us whether sulfite bound to carbonyl compounds is able to protect various organic compounds toward a peroxidatic oxidation, as free sulfite does. However, the increase in the concentration of sulfate showed that at least a fraction of the carbonyl bisulfite addition compounds were oxidized in a coupled reaction.

SO_2 + Beer Without Ascorbic Acid

This is, of course, the more general case, the one we are most concerned with. Beer contains oxidizable components, most of them polyphenolic in nature. However, as the oxidation of polyphenols is a very slow phenomenon, one can no longer consider that the spontaneous oxidation of sulfite proceeds at a negligible speed in comparison to that of polyphenols. Several questions arise: 1) In the presence of excess O_2 (conditions of the model trials), do substrates oxidize independently of one another? 2) Is there any competition for certain common catalysts (metal ions, for example)? 3) In the presence of limited amounts of O_2 (usual conditions for packaged beer), does competition for O_2 occur? 4) Is there a coupling similar to that which has been proved to exist with AH_2? In other words, does the oxidation of polyphenols induce that of sulfite?

In this case, the experimental approach is much more difficult and the methods used for the study of the oxidation of a mixture $AH_2 + SO_2$ fail to give a satisfactory picture of the phenomenon. Indeed, because the speed of O_2 fixation is very slow, one can at best study the initial stages of oxidation.

The manometric method shows irregularities and no longer gives us an exact measure of the O_2 absorbed.

The mechanisms of oxidation of phenolic compounds by O_2 cannot be simply deduced from the results of oxidation by other oxidizing agents. We were able to show, for example (9), that the reducing power of catechin or quercetin ($DPFe_3$ method) was only slightly affected by a peroxidasic oxidation (H_2O_2 + peroxidase [8]), whereas the tanning power, and accordingly the ability to induce hazes in beer, was strongly increased.

Most of the sulfite added to beer gives bisulfite addition compounds in the first few minutes. During the subsequent oxidation, both free and bound sulfite participate in the reaction. Because the equilibrium between the two forms is slow and probably incomplete, it follows that the decrease in the concentration of free sulfite is no longer unequivocally related to the oxidation. Besides, there is no proof that the compounds resulting from oxidation of polyphenols are able to bind free sulfite in significant amount.

The nephelometric determination of sulfate actually gives the amount of SO_2 oxidized, but it would be necessary to accurately estimate the amount of O_2 consumed, and, as we stated, the manometric method failed. The formation of peroxides and their accumulation in aqueous solutions of polyphenolics exposed to the air during several months (10), allows certain phenolic compounds to display a catalytic role in the oxidation of sulfite. These peroxides are indeed instantaneously destroyed by the addition of sulfite. Oxidation of sulfite coupled to that of polyphenols cannot be excluded. Many years ago, research with wines showed that, according to the circumstances, the gallic tannin could either enhance the oxidation of SO_2 or play the role of an antioxidant. The results of Ribereau-Gayon and other authors cited in his book (17) teach us that simultaneous oxidation of SO_2 and phenolic compounds always occurs. The ratio between O_2 consumed and SO_2 oxidized can vary within broad limits according to the conditions. The results depend on the presence of a metallic catalyst, on the ratio of iron to copper, on the type of wine, and on the concentration of SO_2. No general rule can be found so far.

An increase of SO_2 concentration slows down the browning of polyphenolic solutions exposed to air. But this does not mean that the oxidation of the polyphenols is completely suppressed. What was said about wines, in which the phenomenon is easier to demonstrate because the concentrations of both phenolics and SO_2 are higher than in beers, is probably still valid for beers.

But a quantitative balance sheet would require the accurate determination of O_2 consumed and the amount of total sulfite (not only that of free SO_2). So far there is no quick micromethod for the determination of total SO_2.

DISCUSSION

Determination of Free SO_2

Use of an oxidizing agent like iodine for determining the free sulfite in a natural product is based on the fact that this reagent reacts instantaneously with the free sulfite. However, one should not overlook the fact that it reacts, though more slowly, on the labile bisulfite addition compounds, and may react, more slowly still, on the strongly bound sulfite. Hence the result depends on both the excess of reagent and the duration of its contact with the medium.

In order to keep its interference in the titration of free SO_2 to a minimum, the excess of iodine must be very low and the contact time must be as short as possible. Because some of the present components display reducing power toward the oxidizing agent (for example melanoidins or reductones), a blank titration is usually made on a sample in which all the free sulfite is blocked by addition of an excess of acetaldehyde (14–16). H_2O_2 does not react under the conditions described above.

The entrainment of free SO_2 by an inert gas at room temperature can only be efficient if the medium to be titrated has first been strongly acidified to insure that all the free sulfite is in the SO_3H_2 form. The acidification leads to a shift in the equilibrium of free and bound SO_2, especially in the case of labile aldehyde bisulfite compounds.

The utilization of higher temperature (distillation) leads to a similar result. Further, volatile aldehydes can also be entrained and then recombine in the condenser (we know that the combination is rapid) before the oxidizing agent into which the condensed products are received (generally H_2O_2) can oxidize all of the SO_2 present.

The strong dilutions that are sometimes necessary (eg, with the rosanilin method) also lead to a shift in the free/bound SO_2 equilibrium, again chiefly affecting the labile bisulfite compounds.

Antioxidant Properties

When one considers the protective role that combined SO_2 is able to play toward other oxidizable substances of the medium, one must not forget that the sluggishness of dissociation of some SO_2 addition compounds, which is useful for an analytical distinction

between free and bound SO_2, is not synonymous with a total lack of reactivity.

It is surely not reasonable that the same amount of oxidizing agent that reaches the medium at a very slow speed, as happens during the aging of wines in wood vessels through which the air diffuses very slowly, is equivalent to that made available at high concentration in a short time, as happens during bottling or pouring off in the air.

For the same amount of oxygen in contact with the beverage, different results should be expected. The protective effect toward phenolic substances should be more efficient in the first case than in the second. Simply extending this reasoning to beer, without further experimental support, would surely be premature.

Finally, the possibility of wholly irreversible addition compounds even in the most extreme conditions cannot be excluded. Brenner et al (2) reports that Nichols and Reed, working on sulfited dried fruits, found that SO_2 was still released after 5 hr of distillation in strongly acidic medium.

Hence it is questionable whether such strongly bound "sulfite" can play any role as an antioxidant, even in very slow oxidations.

In the same context, only a small part of the anthocyanins of red wines that are decolorized by SO_2 can be regenerated on oxidation by H_2O_2. Those reaction compounds probably cannot be considered true bisulfite addition compounds. The concept of equilibrium between free and bound SO_2 obviously cannot be applied to such a case.

CONCLUSION

The graphic presentation of the equilibrium between free and bound sulfite allowed us to pinpoint the practical meaning of the dissociation constant $K_{A \cdot SO_2}$ of a bisulfite addition compound $A \cdot SO_2$ and to illustrate the effect of dilution as well as that of a variable aldehyde/SO_2 ratio on the percentage of free sulfite.

The free sulfite fraction depends to a large extent on the method used for its determination, especially on the nature and concentration of the oxidizing agent, on the time of the reaction, on eventual dilutions, and on changes in temperature and pH.

The use of hydrogen peroxide, which shows negligible reactivity on the components of the natural medium (beer, wine, etc.) when used in very small excess and during short times, allows the selective oxidation of free SO_2 in such a medium.

Utilization of the colorimetric MB method for the selective determination of residual H_2O_2 permits the possibility of precisely determining the free sulfite and making a kinetic study of its binding on the carbonyl components of beer.

The binding of SO_2 is a rapid phenomenon. The nonvolatile fractions have the highest combinating power.

In a mixture of sulfite plus ascorbic acid, the oxidation of sulfite is coupled with that of ascorbic acid. As soon as it is formed, dehydroascorbic acid binds one equivalent of sulfite to give a stable addition compound.

A portion of the sulfite engaged in the formation of addition compounds with carbonyl substances can be oxidized in coupled reactions and, accordingly, can contribute to the elimination of O_2 present in a complex medium.

However, in the presence of sulfite, the speed of O_2 consumption becomes extremely low, even in the presence of easily oxidizable substances like ascorbic acid. The reason was not clearly elucidated.

The presence of free or bound SO_2 does not suffice for completely excluding the oxidative polymerization of anthocyanogens; this was shown in model trials with procyanidins (B_3 or trimers).

A deeper knowledge of the nature of the bound sulfite is an urgent prerequisite for a better understanding of the whole problem.

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