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I am submitting herewith a thesis written by Tina L. Knox entitled "Evaluation of Selected Compounds in Fruit Juices as Sodium Metabisulfite Replacements or Adjuncts." I have examined the final copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Technology and Science.

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4

EVALUATION OF SELECTED COMPOUNDS IN FRUIT
JUICES AS SODIUM METABISULFITE
REPLACEMENTS OR ADJUNCTS

A Thesis
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Tina L. Knox
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DEDICATION

This manuscript is dedicated to my parents, Mr. and Mrs. Arthur F. Knox.

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ABSTRACT

Sodium metabisulfite (sulfite), butylated hydroxyanisole (BHA), potassium sorbate (sorbate) and tertiary butylhydroquinone (TBHQ) were evaluated for their antimicrobial effects on Saccharomyces cerevisiae and Saccharomyces cerevisiae NRRL Y567 in apple and grape juice. Combinations of sulfite with BHA, BHA with sorbate and sulfite with sorbate were tested to determine if the compounds interacted in an additive, synergistic or antagonistic manner.

A modification of the tube dilution technique outlined by Barry (1976) was used to evaluate antimicrobial efficacy. Tubes of the appropriate juice containing increasing concentrations of an antimicrobial or antimicrobial combination were inoculated with a 24 hr yeast culture grown in the juice. These tubes were then incubated 24 hr at 32°C. Tubes showing no visible growth after this time were considered to contain a concentration of antimicrobial(s) which was inhibitory to the yeast. Tubes with antimicrobial free juice were then inoculated with juice from the tubes showing no growth and incubated as before. Antimicrobial concentrations that prevented visible growth in these tubes after this additional 24 hr incubation were considered to be lethal to the yeast.

The individual antimicrobials inhibited S. cerevisiae in apple juice at the following concentrations: 80 µg/ml sulfite, 160 µg/ml BHA, 500 µg/ml sorbate and 600 µg/ml TBHQ. In grape juice against this yeast, the inhibitory concentrations were 80 µg/ml sulfite,

140 $\mu\text{g/ml}$ BHA, 600 $\mu\text{g/ml}$ sorbate and 900 $\mu\text{g/ml}$ TBHQ. When these compounds were used against S. cerevisiae NRRL Y567 in apple juice, levels of 100 $\mu\text{g/ml}$ sulfite, 120 $\mu\text{g/ml}$ BHA, 400 $\mu\text{g/ml}$ sorbate and 600 $\mu\text{g/ml}$ TBHQ effectively inhibited growth. The corresponding values for grape juice were 100 $\mu\text{g/ml}$ sulfite, 160 $\mu\text{g/ml}$ BHA, 400 $\mu\text{g/ml}$ sorbate and 1000 $\mu\text{g/ml}$ TBHQ. The lethal concentration in apple and grape juice against S. cerevisiae for sulfite was 80 $\mu\text{g/ml}$ and for BHA was 160 $\mu\text{g/ml}$. Against S. cerevisiae NRRL Y567, the lethal concentration in both juices for sulfite was 120 $\mu\text{g/ml}$ and for BHA was 160 $\mu\text{g/ml}$. None of the concentrations of sorbate or TBHQ were lethal to the yeast.

In the combination testing, all combinations proved to be additive ($P>0.05$) in nature in both juices and with both organisms, except for the sulfite-sorbate combination in grape juice against S. cerevisiae NRRL Y567. This interaction was considered to be antagonistic in nature since higher concentrations of sulfite were needed to cause inhibition after 24 hr than in other sulfite-sorbate combinations. It was concluded, that combining the antimicrobials would allow for a reduction in sulfite concentration but no significant gain in antimicrobial activity would occur.

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CHAPTER I

INTRODUCTION

Sulfites are added to many foods such as purees, syrups, juices and dehydrated fruits and vegetables to control the growth of bacteria, yeasts and molds. The sulfites also play an important role in preventing refermentation in wines and fermented ciders (Chichester and Tanner, 1972; Splittstoesser, 1982). In addition to serving as an antimicrobial, the sulfites also prevent enzymatic browning in fresh and processed fruits and vegetables (Nolan, 1983; Roberts and McWeeny, 1972).

Recently there has been great concern over the toxicological safety of sulfites as a food additive, especially to asthmatic persons (Anonymous, 1982c). Although searches for mutagenic or carcinogenic affects from dietary sulfites have proven negative, many people have been reported to have suffered from feelings of nausea, headaches and difficult breathing after consuming foods containing sulfites (IFT, 1975; Nolan, 1983).

The currently approved antimicrobial, potassium sorbate, along with two phenolic antioxidants, butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ), are possible replacements for sulfites in food products. Sorbic acid and its potassium salt have been used in wines, other fermented and non-fermented beverages, and dried fruits to prevent microbial spoilage (Sofos and Busta, 1981). The phenolic antioxidants, BHA and TBHQ, have also been shown

to have antimicrobial activity against a wide variety of microorganisms (Branen et al., 1980). The dual functionality of these phenolic antioxidants makes their use as sulfite alternatives quite feasible.

The objective of this research was to evaluate the antimicrobial efficacy of BHA, TBHQ and potassium sorbate as possible replacements for or adjuncts to sodium metabisulfite.

CHAPTER II

REVIEW OF LITERATURE

1. SACCHAROMYCES CEREVISIAE

The genus Saccharomyces is a heterogenous group of organisms with spheroidal, ellipsoidal, cylindrical, thread-like or elongate cells with pseudomycelia (Banwart, 1979; Beneke and Stevenson, 1978). The colonies are generally cream or white with a typical yeasty odor (Banwart, 1979). When grown in a liquid media, bottom growth usually occurs, but some strains tend to collect on the surface and are called top fermentors (Banwart, 1979; Pelczar et al., 1977).

Saccharomyces cerevisiae is one of the most beneficial yeasts from this genus (Reed and Peppler, 1973). There are at least 1000 separate strains of this organism. In the current classification system, bakers yeasts, wine yeasts and champagne yeasts are all considered to be strains of S. cerevisiae (Beneke and Stevenson, 1978).

Saccharomyces cerevisiae cells measure 2.5-10.5 μm in width, and 4.5-20 μm in length. Filamentous strains may attain lengths greater than 30 μm (Reed and Peppler, 1973). The thin cell wall is composed of 60-65% of the polysaccharides glucan and mannan, 6-8% protein, 1-2% chitin, 8.5-13.5% lipid and 11.5-24.5% of organic materials (Pelczar et al., 1977; Ponte and Tsen, 1978).

Saccharomyces cerevisiae tend to metabolize oxidatively but can also ferment (Pelczar et al., 1977; Ponte and Tsen, 1978). They

do not utilize nitrate or lactose but many can ferment simple monosaccharides and can hydrolyze sucrose and maltose to simple sugars (Banwart, 1979; Ponte and Tsen, 1978).

The optimum growth temperature for S. cerevisiae is approximately 28-32°C (Humphreys and Stewart, 1978). The minimum water activity for growth of the organism is 0.90-0.94, while their minimum growth pH is 2.0-2.4 and optimum pH is 4.0-5.0 (Banwart, 1979).

The ability of some species of S. cerevisiae to produce alcohol and carbon dioxide makes this organism one of the most useful wine yeasts (Banwart, 1979). S. cerevisiae imparts a fruit-like flavor to wine, has a high tolerance for alcohol, and will ferment sugar to a low content producing 14-18% alcohol (Pederson, 1979; Pelczar et al., 1979).

These organisms are associated with a variety of foods and can cause spoilage in fruit and fruit products, dairy products, and fermenting foods (Banwart, 1979). Often wild strains of S. cerevisiae contaminate the pure cultures employed by brewers and wine makers and cause off flavors, turbidity, color changes and sedimentation in their product (Reed and Pepler, 1973; Splittstoesser, 1978). No pathogenicity has been established for any species of these yeast (Banwart, 1979).

2. SULFITES

The sulfites in various forms have been added to food materials as preservative agents since the times of the ancient Greeks

(Wedzicha, 1981a; IFT, 1975). Sulfur dioxide is used as a food additive to inhibit non-enzymatic browning, various enzyme catalyzed reactions (e.g., enzymatic browning), and microorganisms, and is used as an antioxidant and reducing agent (Roberts and McWeeny, 1972).

Chemical and Physical Properties

Sulfur dioxide is a colorless, noncombustible gas with a pungent odor, and is fairly soluble in water (Lueck, 1980; Ough, 1983). It is rarely added, as such, to food but is usually added in a salt form such as sodium sulfite, sodium hydrogen sulfite, sodium metabisulfite, or potassium metabisulfite (Lueck, 1980; Wedzicha, 1981a). Table 1 lists some forms of sulfur dioxide, and their molecular weights. Bisulfites exist in solution form only, and upon drying, their anhydrides are the metabisulfites (Anonymous, 1982b; Lueck, 1980; Ough, 1983). It is useful to have the sulfur dioxide in salt form because these dry salts are easier to store and less of a problem to handle than gaseous or liquid sulfur dioxide (Ough, 1983). The metabisulfites are more stable than the bisulfites which in turn are more stable than the sulfites (Chichester and Tanner, 1972; Ough, 1983). The particular chemical agent used for the sulfiting treatment is primarily a matter of convenience, because when dissolved in water, the sulfites, metabisulfites and sulfur dioxide form sulfurous acid, bisulfite ions and sulfite ions (IFT, 1975).

When sulfur dioxide is dissolved in water, sulfurous acid is formed and dissociates as follows (King et al., 1981):

TABLE 1. Sulfur Dioxide-Containing Chemicals

Chemical	Formula	Molecular Weight
Sulfur dioxide	SO ₂	64.04
Potassium sulfite	K ₂ SO ₃	190.10
Sodium sulfite heptahydrate	Na ₂ SO ₃ *7 H ₂ O	252.15
Potassium bisulfite	KHSO ₃	120.16
Sodium bisulfite	NaHSO ₃	104.06
Potassium metabisulfite	K ₂ S ₂ O ₅	222.34
Sodium metabisulfite	Na ₂ S ₂ O ₅	190.10

SOURCE: Ough, 1983; Lueck, 1980.



Many researchers feel that sulfurous acid does not exist in aqueous solutions (IFT, 1975; Macris and Markakis, 1974). According to King et al. (1981), the greatest percent of the SO_2 may not exist in aqueous solution as sulfurous acid, but it would be impossible to produce enough H^+ ions to make an acid as strong as sulfurous without dissociation as H_2SO_3 . The actual form in which the additive may be present and exert its preservative action is a function of pH and concentration (Wedzicha, 1981a). King et al. (1981) reported that the correct dissociation constants for sulfur dioxide are 1.7×10^{-2} and 6.31×10^{-8} . Other researchers have reported differing pH values (IFT, 1975; Ough, 1983).

Sulfites form addition compounds with carbonyl compounds such as aldehydes, ketones and sugars (Gunnison, 1981; IFT, 1975; Lueck, 1980). Conditions are highly conducive to their formation, especially in the pH range of 3-5 in which sulfur dioxide is used the most (Lueck, 1980). The linkage of sulfurous acid and carbonyl compounds in foods generally reduces or neutralizes the antimicrobial action of the acid against yeasts. The acetaldehyde addition compound is slightly active against certain bacteria but the addition compounds with sugars are ineffective (Lueck, 1980).

Sulfite ions also cleave sterically available disulfide bonds in proteins resulting in the formation of a thiol and an S-substituted thiosulfate (IFT, 1975). A specific reaction leading to an

antinutritive effect caused by sulfites is the cleavage of thiamine (vitamin B₁) into two fragments which no longer possess vitamin activity (IFT, 1975; Wedzicha, 1981b). Sulfite destroys thiamine by breaking the bond between the pyrimidine and the thiazole portions of the molecule (Lueck, 1980). It is highly probable that all thiamine dependent enzyme reactions are inhibited by sulfite. This reaction has a maximum rate in the region of pH 5-6 and is a potential disadvantage of the use of sulfur dioxide as a food additive (Wedzicha, 1981b).

Safety and Metabolism

When sulfur dioxide is administered orally, the LD₅₀ for the rat is 1000-2000 mg sulfur dioxide per kg of body weight (Lueck, 1980). A relatively low acceptable daily intake of 0.70 mg/kg of body weight has been established for sulfur dioxide by the World Health Organization (IFT, 1975; Lueck, 1980). Concern over the possible toxicity of sulfites in food goes back to 1896 when an article was published on the possible hazards of sulfur dioxide. Since 1976, the Food and Drug Administration has investigated over 100 reported cases of adverse reactions to sulfites (Anonymous, 1982c; Nolan, 1983). Human reactions to sulfur dioxide vary widely. While most people can tolerate up to 4 grams of sulfite daily without untoward effects, others complain of headaches, nausea, diarrhea, a feeling of satiation, anaphylactic shock, abdominal pain, dizziness and hives after ingesting only very small quantities of

sulfite (Lueck, 1980; Nolan, 1983). Asthmatics as a group seem to show the most sensitivity with an estimated 5-11% of all asthmatics affected. In addition, approximately 25% of the reported cases of sensitivity to sulfites have been people with no known history of asthma (Noland, 1983). Searches for mutagenic or carcinogenic effects from dietary sulfites have been unsuccessful in nearly all cases (IFT, 1975).

Presumably sulfites added to food materials are metabolized in the same way as the sulfur containing amino acids, and in the amounts normally consumed contribute less than 96 mg to the normal daily sulfate production and excretion (IFT, 1975). The usual diets in the U.S. provide more of the sulfur containing essential amino acids cystine and methionine than the body can utilize and retain. The normal metabolic process of the body can convert the excess sulfur in the amino acids first to sulfites and then with the aid of the enzyme sulfite oxidase, to sulfates which are excreted in the urine (Chichester and Tanner, 1972; IFT, 1975; Lueck, 1980).

Regulatory Status

Sulfur dioxide, some sulfites, bisulfites and metabisulfites are permitted as food preservatives in virtually all countries (Lueck, 1980). Since 1959, sulfur dioxide, sodium sulfite, sodium and potassium bisulfite and sodium and potassium metabisulfite have been listed as Generally Recognized As Safe (GRAS) by the FDA (Anonymous, 1982a; Chichester and Tanner, 1972; Nolan, 1983). There

is a proviso however that sulfites cannot be used in foods which are substantial sources of thiamine (Anonymous, 1982a; Chichester and Tanner, 1972).

In general, the approval of sulfur dioxide is restricted to foods of vegetable origin and beverages including wine (Lueck, 1980). Only a few countries allow the use of sulfites to treat meat, because its use restores a bright color and appearance of freshness to the meat and this is considered to be an act of consumer deception (Chichester and Tanner, 1972; Lueck, 1980).

Applications

The major use of the various forms of sulfite is in the fruit, wine and non-fermented beverage industries to prevent microbial spoilage (Ough, 1983). They are used in wine making as selective antibacterial agents which do not inhibit the desired development of yeast (IFT, 1975). In some applications of sulfiting, the anti-microbial action is concurrent with other desirable effects such as the prevention of enzymatic and non-enzymatic discoloration and inhibition of chemically induced color and vitamin losses (Lueck, 1980; Wedzicha, 1981a).

Any raw, dried, frozen or canned vegetable or fruit that is subject to enzymatic or non-enzymatic browning can benefit by proper treatment with sulfite compounds. Vegetables such as peas, carrots, beans, cabbage, potatoes and tomatoes have a more stable color and less deterioration if treated with these compounds (IFT, 1975; Ough, 1983).

Sulfite levels in processed foods range from 1 to about 3000 ppm on a dry weight basis. Dehydrated, light colored fruits such as apples, apricots, bleached raisins, pears and peaches contain the greatest amounts. Dehydrated vegetables and prepared soup mixes range from a few hundred to about 2000 ppm (IFT, 1975).

Among beverages for which sulfur dioxide may be used, the most important is wine (Lueck, 1980). A worldwide average for sulfites in wines is 100-400 ppm sulfite, and for beers 2-8 ppm. The maximum amount of sulfur dioxide legally permitted by the FDA is 350 ppm (IFT, 1975). Sulfur dioxide is added to fresh grape must at a level of 40-50 mg/liter to prevent the growth of acetic acid bacteria, wild yeasts and molds. At this level the sulfur dioxide does not damage the selected wine yeast (Chichester and Tanner, 1972; Lueck, 1980). Higher concentrations of sulfur dioxide are used to prevent any further fermentation (Lueck, 1980).

Antimicrobial Activity

The antimicrobial action of sulfurous acid and its salts is strongly pH dependent (Lueck, 1980; Macris and Markakis, 1974). The dissolved sulfur dioxide gas and undissociated sulfurous acid possess the most effective antimicrobial action (Lueck, 1980; Ough, 1983). King et al. (1981) reported that the undissociated sulfurous acid was the only effective molecular species in killing yeast and the combined SO_2 has no killing effect.

When considering the antimicrobial activity of sulfur dioxide and its salts, three main groups of microorganisms are of interest

in the more acid beverages and fruits. These are acetic acid-producing and malolactic bacteria, fermentation and spoilage yeasts, and fruit molds (Ough, 1983; Splittstoessor, 1982). Sulfurous acid inhibits yeasts, molds and bacteria, but is most effective against acetic and lactic bacteria (Chichester and Tanner, 1972; Ough, 1983).

Most bacteria are inhibited even at pH 6 by 50 to 200 ppm sodium sulfite (Chichester and Tanner, 1972; Lueck, 1980). Many yeast strains react differently to sulfur dioxide. Most yeasts are inhibited by 80-240 ppm of sulfur dioxide, but sulfur dioxide will not always inhibit some strains of yeast. Torulopsis and Saccharomyces appear to be the most tolerant yeast with some 30 strains of Saccharomyces being characterized as resistant to this compound (Ough, 1983). Lueck (1980) reported that S. cerevisiae was usually inhibited by 80-160 sodium sulfite ppm (50% sulfur dioxide) at pH 4.0. King et al. (1981) reported that a concentration of 100 ppm sulfur dioxide was lethal to this organism at pH 3.5. There is very little difference in the inhibitory concentrations reported by these two researchers when the sulfur dioxide equivalents are compared. In similar concentrations, sulfur dioxide is as effective against molds as it is against yeast (Lueck, 1980). Splittstoessor and Mattick (1981) reported that 200 ppm sulfur dioxide in grape juice appeared to completely inhibit mold growth and prevented fermentation in most cases.

Owing to its good antibacterial effect, sulfur dioxide is frequently combined with the preservatives sorbic acid and benzoic

acid, which have more of a fungistatic action (Lueck, 1980; Splittstoesser, 1982). This improves the antimicrobial spectrum of both groups of preservatives giving protection against both lactic acid bacteria and yeast (Lueck, 1980).

Mechanism of Inhibition

The mechanism by which antimicrobials inhibit microorganisms generally falls into one or more of three general categories: (1) reaction with the cell membrane to cause increased permeability with resultant loss of cellular constituents, (2) inactivation of essential enzymes, and (3) destruction or functional inactivation of genetic material (Davidson and Branen, 1981).

The antimicrobial action of sulfur dioxide has been attributed to interference with the enzyme structure of the cell (Lueck, 1980; Ough, 1983). The secondary and tertiary structure of many enzymes are due to disulfide links, and Wedzicha (1981b) suggests that cleavage of these bonds is one way in which enzymes are inactivated by sulfites. Damage to structural proteins in microbial cells could also form part of the basis for its antimicrobial effect (Wedzicha, 1981b).

3. SORBATES

The antimicrobial properties of sorbic acid were first discovered in the 1940's (Lueck, 1980; Sofos and Busta, 1981). During the last 30 years, sorbate has been tested and widely used in the preservation of various food products throughout the world.

Chemical and Physical Properties

Sorbic acid is a straight chain beta-unsaturated trans-trans, 2-4-hexadienoic monocarboxylic aliphatic acid and has the molecular formula $\text{CH}_3\text{-CH=CH-CH=CH-COOH}$ (Chichester and Tanner, 1972; Sofos and Busta, 1981). The carboxyl group of sorbic acid reacts readily and forms salts and esters. The salts of sorbic acid, especially the potassium salt (molecular weight 150.22), are very important in food applications due to their high solubility in water (Sofos and Busta, 1981, 1983). The low water solubility of the acid form is its primary disadvantage (Sofos and Busta, 1981). The solubility of sorbic acid in water increases with increasing pH and temperature. Increased concentrations (>10%) of soluble food components such as glucose, sucrose and NaCl reduce the solubility of sorbic acid in water (Chichester and Tanner, 1972; Sofos and Busta, 1981). In vegetable oil, the acid is more soluble than the potassium salt. An increased fat content will lower the amount of sorbic acid in the aqueous phase where it is needed for microbial control (Sofos and Busta, 1981).

Safety and Metabolism

A considerable amount of research has dealt with the toxicity, metabolism and stability of sorbate as a preservative. Early in its use, sorbate was classified as relatively non-toxic. The LD_{50} for sorbic acid with rats is about 10 g/kg of body weight. An Acceptable Daily Intake for sorbate of 25 mg/kg of body weight was

established by the World Health Organization, which is the highest stipulated for any food preservative (Sofos and Busta, 1981). Sorbate is metabolized like other fatty acids in the mammalian body to yield CO_2 and water (Beuchat 1981b; Sofos and Busta, 1981).

Regulatory Status

In the U.S., sorbate is a GRAS substance and it may be used in any food which allows preservatives. No upper limits are imposed for foods not under a Federal Standard of Identity. Levels of incorporation into the more than 70 products having Standards of Identity that allow use of sorbates vary from 0.01 to 0.30% (Chichester and Tanner, 1973; Sofos and Busta, 1981).

Applications

Sorbic acid and its potassium salt are the most widely used forms of the sorbates (Sofos and Busta, 1981). They are relatively tasteless and odorless (Chichester and Tanner, 1972). Sorbate is utilized as a food preservative in dairy products, bakery products, fruit and vegetable products including wines and fruit juices, condiments, and certain fish and meat products (Chichester and Tanner, 1972). In fruit juice processing, sorbic acid is used mainly in conjunction with small quantities of sulfur dioxide to protect the product from oxidation and enzymatic and microbial spoilage (Lueck, 1980). The use of sorbic acid is also highly important in wines where it is used to stabilize wine against yeast refermentation (Lueck, 1980; Sofos and Busta, 1983). Here again, sorbate is used

in combination with sulfite to inhibit enzymatic and oxidative changes and bacterial fermentation (Chichester and Tanner, 1972; Sofos and Busta, 1983).

Antimicrobial Activity

Sorbate has been shown to inhibit growth of yeasts, molds and many bacteria, however it is most effective against yeasts and molds (Chichester and Tanner, 1972; Sofos and Busta, 1981). Certain mold species are resistant to sorbic acid and occasional mold spoilage occurs in foods preserved with this compound (Sofos and Busta, 1981). The yeast and filamentous fungi are inhibited in media containing 0.1% sorbic acid at pH 4.5 (Chichester and Tanner, 1972). Lueck (1980) reported minimum inhibitory concentrations of sorbic acid for S. cerevisiae of 25 ppm at pH 3 and for Saccharomyces species at 30-100 ppm at pH 3.2-5.7 (Lueck, 1980).

It has been reported that low intracellular concentrations of sorbic acid are metabolized by yeasts (Sofos and Busta, 1983). While at high levels, yeast metabolism and growth is suppressed. Warth (1977) found that S. bailii was resistant to sorbic acid and suggested that resistance resulted primarily from an inducible system which transported the compound out of the cell. He also theorized that this mechanism was induced when the organism was grown in the presence of low concentrations of the preservative. Bills et al. (1982) reported that S. rouxii cells preconditioned in 0.1% sorbate also showed an increased resistance to sorbate.

Only undissociated sorbic acid has any antimicrobial activity, therefore, sorbate is more effective at pH values approaching its pKa of 4.75. At this pH, 50% of the acid is in the effective undissociated form (Sofos and Busta, 1981). Since its activity increases as the pH of the medium declines, sorbate is more effective in acid foods where yeasts and molds are the primary spoilage agents (Beuchat, 1981b; Chichester and Tanner, 1972).

Soluble food components such as sugars and salt increase the ratio of fat to water of the sorbate partitioning, which indicates that a lower level of sorbate remains in the water phase of the system to act as an antimicrobial agent (Sofos and Busta, 1983). In contract to this, studies have indicated that salts and sugars act synergistically with sorbate and increase the antimicrobial effects (Beuchat, 1981a; Chichester and Tanner, 1972; Costilow et al., 1955; Gooding et al., 1955; Sheneman and Costilow, 1955; Sofos and Busta, 1981). Many researchers have found synergistic interactions among sorbate, pH level, salt and sugar concentration (Costilow et al., 1955, 1956, 1957; Gooding, 1955; Sofos and Busta, 1981). Restaino et al. (1982 reported that 500 ppm potassium sorbate inhibited S. bailii and acidifaciens and 1000 ppm inhibited growth of S. rouxii and bisporus on acidulated agar.

Many researchers have reported that sorbates exhibit synergistic inhibitory activity with the phenolic antioxidants butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) (Davidson et al., 1981; Lahellec et al., 1981). Robach and Stateler (1980) found that

combinations of 0.1% sorbate and 25 ppm TBHQ resulted in inhibition Staphylococcus aureus after 12 hours at 37°C and combinations of 0.2% sorbate and 100 ppm BHA synergistically inhibited growth of this organism. In contrast, Ahmad and Branen (1981) found no apparent synergism between BHA and sorbate against Aspergillus flavus, although a combination of sorbic acid and BHA gave a greater percentage of inhibition than did BHA alone.

Mechanism of Inhibition

Inhibition of microbial cells by sorbate is generally static in nature and only rarely results in inactivation of the cells (Sofos and Busta, 1983). The antimicrobial action of sorbic acid has been attributed to several causes. Researchers have reported that sorbates inhibit various enzymes in the microbial cell, such as those involved in carbohydrate metabolism (Lueck, 1980; Sofos and Busta, 1983). Sorbic acid is known to form covalent bonds with the sulfhydryl groups of enzymes thereby inactivating them (Whitaker, 1959; York and Vaughn, 1964). Sorbic acid is known to act against catalase positive microorganisms by influencing the enzymes catalase and peroxidase (Beuchat, 1981b, Lueck, 1980). York and Vaughn (1964) and later Sofos and Busta (1983) suggested that sorbates uncouple oxidative phosphorylation.

4. PHENOLIC ANTIOXIDANTS

Phenolic compounds have been used as antioxidants since the 1960's. In 1975, research on the use of phenolic antioxidants

as antimicrobials was begun when Chang and Branen conducted research with butylated hydroxyanisole (Chang and Branen, 1975; Davidson, 1983). Previous to this, BHA, along with other similar compounds such as tertiary butylhydroquinone, had been used strictly as an antioxidant to prevent oxidative rancidity in lipids and lipid-containing foods. Since phenolic compounds were known to possess inhibitory powers against microorganisms, it was reasoned that the phenolic antioxidants might also have antimicrobial properties (Chang and Branen, 1975; Davidson, 1983). These compounds were already approved for use in foods by the Food and Drug Administration, and many researchers felt that testing was warranted to determine if the antioxidant had multiple functions (Davidson, 1983).

Chemical and Physical Properties

Butylated hydroxyanisole exists as the 2- and 3-isomers of tertiary-butyl-4-hydroxyanisole with the 3-isomer predominating. The molecular weight of BHA is 180.0. Tertiary butylhydroquinone is a metabolite of BHA and has a molecular weight of 166.22 (Davidson, 1983; Surak, 1977). These compounds are fairly soluble in lipids but insoluble in water. The primary function of these compounds in foods is to prevent autoxidation of unsaturated lipids in foods (Davidson, 1983).

Safety and Metabolism

The toxicology and metabolism of BHA and TBHQ has been studied extensively and was reviewed by Davidson (1983). An LD₅₀ of

2000 mg/kg for BHA has been reported to be average for most animals. The LD₅₀ for TBHQ in rats has been determined to be 700-1000 mg/kg. Metabolism of BHA and TBHQ takes place in the liver and the metabolites are excreted in the urine.

Regulatory Status

BHA is considered to be GRAS for use in foods at a maximum concentration of 0.02% based on the weight of oil or fat content of the product (Stuckey, 1972). After a recent GRAS review, this compound was placed in a class 3 status and it has been recommended that additional studies be performed on its toxicological safety, but use of the compound is still allowed until these safety studies have been completed (DHHS, 1980; Davidson, 1983).

Butylated hydroxyanisole and tertiary butylhydroquinone are permitted as direct food additives for specific food uses (Code of Federal Regulations, 1980). The use concentrations for BHA range from 2.0 ppm in beverages and dry mix desserts to 1000 ppm BHA in active dry yeast. Other uses include incorporation into dry breakfast cereals and dehydrated potatoes. For TBHQ, the regulation permits use alone or with BHA and BHT at a concentration not to exceed 200 ppm, based on the weight of lipid in the food (Code of Federal Regulations, 1980). TBHQ has also been approved for use in a variety of meat products (Davidson, 1983).

Antimicrobial Activity

A lack of standardization among testing methods, strains, diluents, incubation conditions and media in antimicrobial studies using phenolic antioxidants has resulted in a wide variation in the inhibitory concentrations found. This must be kept in mind when comparing studies (Davidson, 1983; Eubanks and Beuchat 1982b; Sands and Bennet, 1966).

The phenolic antioxidants have been tested against several gram negative and gram positive bacteria. Studies have indicated that, in general, molds and gram positive bacteria are more susceptible to BHA than gram negative bacteria (Branen et al., 1980; Chang and Branen, 1975; Davidson, 1983). Chang and Branen (1975) reported that BHA had a profound affect against Aspergillus parasiticus, Staphylococcus aureus, Eschericia coli and Salmonella typhimurium. The research that is available has indications that TBHQ is extremely effective against gram positive bacteria, especially S. aureus, but is much less effective against gram negative bacteria (Davidson, 1983).

The effects of the phenolic antioxidants on fungi have also been evaluated. Ahmad and Branen (1981) found that 150-200 ppm BHA effectively inhibited the growth of Geotrichum candidum and several species of Aspergillus and Penicillium. Lin and Fung (1983) and Fung et al. (1977) indicated that aflatoxin production by Aspergillus in the presence of BHA, TBHQ or a combination of BHA and TBHQ was reduced and concluded that these antioxidants used in combination acted synergistically in both a media and food product.

Compared to information available on molds, little is known about the effects of food antioxidants on yeasts. Eubanks and Beuchat (1982b) found that 150 ppm BHA caused a marked inhibition of three strains of S. cerevisiae and at 300 ppm no colony formation was observed. Colony formation by the same three strains of S. cerevisiae were affected by 200-400 ppm TBHQ, but levels greater than 500 ppm were required to inhibit colony formation completely (Eubanks and Beuchat, 1982b).

The effect of pH on the antimicrobial activity of BHA has been shown to be dependent upon the microorganism being tested. Butylated hydroxyanisole was found by Ahmad and Branen (1981) to be effective in glucose salts broth over a wide range of pH values and incubation temperatures against Aspergillus flavus. Eubanks and Beuchat (1982b) reported that 150 ppm BHA or 300 ppm TBHQ at a pH of 3 or 7 showed increased inhibitory effects on the rate of growth of S. cerevisiae compared to the effects at pH 4-7. Klindworth et al. (1979) found similar results using BHA against Clostridium perfringens, i.e., inhibition was the greatest at the extremes of pH range tested (5.5-8.5). Stern et al. (1979) found that BHA at 1, 50 and 100 ppm became more effective in preventing growth of Staphylococcus aureus as the pH of the medium decreased from 7 to 6 and the NaCl concentration increased from 5-7%. Differences in results with pH may be traced to different techniques of evaluating activity (Branen et al., 1980; Davidson, 1983).

The presence of lipids in food or test media has been shown to dramatically decrease the effectiveness of both BHA and TBHQ. For example, Klindworth et al. (1979) showed inhibition of Clostridium perfringens by BHA was reduced in the presence of corn oil and Ahmad and Branen (1981) found similar results with BHA against Aspergillus flavus. Rico-Munoz and Davidson (1983) found that the addition of corn oil essentially eliminates the antimicrobial properties of BHA against five organisms including S. cerevisiae, and diminished the activity of TBHQ. The effect of lipids on the activity of BHA could be due to either the solubilization of the BHA into any lipid present or the BHA being "used" up in preventing autoxidation (Branen et al., 1980). Rico-Munoz and Davidson (1983) also found that casein decreased the antimicrobial activity of BHA and to a lesser extent, TBHQ. This decrease in effectiveness was probably due to the binding of the antioxidants by the casein through hydrophobic interactions (Cornell et al., 1971; Rico-Munoz and Davidson, 1983). Minerals such as NaCl have been found to increase the effectiveness of BHA against organisms (Branen et al., 1980).

The influence of temperature on the activity of BHA appears to be dependent upon the organism used in the evaluation. Davidson and Branen (1980) found that 100-200 ppm BHA delayed the growth of Pseudomonas fluorescens at 22°C but totally inhibited growth of the organism at 7°C. In contrast, they found that Pseudomonas fragi grew in the presence of levels up to 400 ppm BHA at both 7 and 22°C. They theorized that lower temperatures decreased the activity of

BHA by reducing its solubility into the lipid portion of the micro-organism. Eubanks and Beuchat (1983) found that the rate of death of S. cerevisiae subjected to the lethal temperature of 50°C was enhanced by as little as 50 ppm BHA and 100 ppm TBHQ.

When used with other antimicrobial preservatives the phenolic antioxidants have generally been reported to demonstrate either an additive or synergistic effect (Davidson, 1983). BHA and TBHQ have been found to exhibit synergistic activity with potassium sorbate against bacteria (Robach and Stateler, 1980). Davidson et al. (1981) found that a sorbate-BHA combination caused a delay in growth initiation of Staphylococcus aureus and that the action of this combination was synergistic. Lahellec et al. (1981) reported that potassium sorbate used in combination with BHA exerted bactericidal and bacteriostatic effects on Staphylococcus aureus and that TBHQ was inhibitory to this organism with or without sorbate. In contrast, Ahmad and Brannen (1981) found no evidence of synergism between BHA and sorbate when used against Aspergillus flavus.

Mechanism of Inhibition

Little work has been done on determining the mechanism of microbial inhibition by phenolic antioxidants. It is most likely that they act on the cytoplasmic membrane of the microorganism in a way similar to the action of other phenolic compounds (Davidson, 1983). Butylated hydroxyanisole has been found to cause leakage of intracellular material from bacteria, apparently as a result of

reactions with the cell membrane (Davidson and Branen, 1979; Degre and Sylvestre, 1983). The mechanism of microbial inhibition might also be related to the destruction or inactivation of essential enzymes and/or genetic material. Surak et al. (1976) and Surak (1977, 1980) suggested that the primary effect of BHA and TBHQ on Tetrahymena was inhibition of DNA, RNA and protein synthesis. Tertiary butylhydroquinone and BHA also inhibited incorporation of ^{14}C -acetate into proteins and lipids (Surak, 1977; Surak and Singh, 1980). Surak and Singh (1980) suggested that BHA altered the rate of synthesis of various lipids in Tetrahymena thereby altering the lipid composition of the cell.

CHAPTER III

MATERIALS AND METHODS

1. MICROORGANISMS

Saccharomyces cerevisiae was obtained from the culture collection of the Food Technology and Science Department of The University of Tennessee, Knoxville. Saccharomyces cerevisiae NRRL Y567 was obtained from the USDA Northern Regional Research Laboratory, Peoria, IL.

The S. cerevisiae was grown for 24 hours at 32°C on yeast extract sucrose (YES) agar slants while S. cerevisiae NRRL Y567 was grown on yeast extract maltose extract (YM) agar slants. Both were stored at 4°C.

2. TEST MEDIA

The test media were commercially purchased apple or white grape juice which did not contain antimicrobials. The pH of the commercial juices was 3.4. The commercial juices were filter sterilized using 0.45 µm membrane filters prior to use. For the combination testing, the commercial white grape juice became unavailable, therefore Thompson white grapes were cold pressed in the laboratory to produce the juice. The laboratory prepared juice (pH 3.5-3.6) was stored in plastic bags, overlaid with nitrogen gas and frozen at -20°C. Prior to use, the juice was centrifuged, decanted and pasteurized at 185°C.

3. TEST COMPOUNDS

Butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) were donated by Eastman Chemical Products, Kingsport, TN. These chemicals were stored in their original containers at 4°C until used. Stock solutions were prepared by dissolving these compounds in 95% ethanol and filter sterilizing through 0.45 µm membrane filters. Solutions of 5% (w/v) BHA and 10% (w/v) TBHQ were prepared prior to each test in volumetric flasks. Potassium sorbate (Monsanto, St. Louis, MO) and sodium metabisulfite (Fisher Scientific, Fair Lawn, NJ) were stored in their original containers at room temperature (21°C) until used. Stock solutions were prepared by dissolving the compounds in distilled water followed by filter sterilization as before. Solutions of 10% (w/v) potassium sorbate and sodium metabisulfite were prepared prior to each test. Concentrations of each antimicrobial were prepared at even intervals in sterile volumetric flasks from the stock solutions to contain up to 120 µg/ml sulfite, 160 µg/ml BHA, 1000 µg/ml TBHQ and 600 µg/ml potassium sorbate.

4. EVALUATION OF CELL GROWTH

Prior to inoculation of the test media, stock cultures were incubated for 24 hr at 32°C in 10 ml of the appropriate juice. The 24 hr culture was then transferred (1 ml) into 50 ml of the juice and grown for an additional 24 hr at 32°C.

Yeast cells were enumerated on YES agar (S. cerevisiae) or YM agar (S. cerevisiae NRRL Y567) from the test juice after 48 hr at 32°C. The initial yeast cell concentrations were approximately 1×10^7 CFU/ml for the apple juice and 3×10^7 CFU/ml for the grape juice.

5. DETERMINATION OF ANTIMICROBIAL ACTIVITY

The technique used to evaluate the antimicrobial activity of the compounds was modified from the tube dilution technique described by Barry (1976). Each antimicrobial was tested alone and in combination to determine their individual and combined inhibitory concentrations and lethal concentrations.

In the individual antimicrobial study, the diluted antimicrobial (0.1 ml) was dispensed into tubes containing 9.8 ml of either the apple or grape juice. The tubes were then inoculated with 0.1 ml of juice which contained the 24 hr yeast culture and incubated for 24 hr at 32°C. Turbidity was used as an indication of growth. The concentration of the antimicrobial in the tubes showing no visible growth after 24 hr was considered to be the inhibitory concentration. One ml of juice from the tubes showing no visible growth was transferred into 9 ml of fresh antimicrobial free juice and incubated for an additional 24 hr. Tubes showing no growth after this additional incubation were considered to contain a lethal concentration of antimicrobial.

In the combination study, combinations of potassium sorbate with BHA, sodium metabisulfite with BHA and potassium sorbate with sodium metabisulfite were tested in both juices with S. cerevisiae and S. cerevisiae NRRL Y567 to determine if the inhibitory and lethal activities of these compounds were additive, synergistic or antagonistic. Tertiary butylhydroquinone was not used in combinations with the other preservatives due to its lack of appreciable inhibitory powers. Lethal concentrations were not established for either combination containing sorbate due to the high concentration of the compound necessary for lethality. For certain comparisons, concentrations were considered to be inhibitory or lethal to the microorganism if greater than half of the replications at a particular concentration were found to show no growth.

6. STATISTICAL ANALYSIS

In the appropriate inhibitory ranges, a minimum of four replications were performed with the antimicrobials used alone and in combination in apple and grape juice against S. cerevisiae and S. cerevisiae NRRL Y567. Analysis of variance (ANOVA) using the General Linear Models Procedure (GLM) was employed to determine if inhibition as measured by the inhibitory concentration was influenced by strain, replications, media or compound (SAS Institute Inc., 1982). Where significant effects were detected ($P < 0.05$), Duncan's multiple range test was performed to identify differences between mean values (SAS Institute., 1982).

The antimicrobial combinations were analyzed by analysis of variance using the Functions of Categorical Response Procedure (FUNCAT) of the Statistical Analysis System (1982) to determine if interactions existed between compounds. If a significant ($P < 0.05$) linear interaction occurred, this was considered to be evidence for either an antagonistic or synergistic influence of the compounds on one another. The chi square analysis from the FUNCAT Procedure was used to develop a prediction equation to generate P values (percentage inhibition) and this data was used with the antimicrobial concentrations to produce three dimensional plots of the data (SAS Institute Inc., 1981).

CHAPTER IV

RESULTS AND DISCUSSION

1. INHIBITION BY THE ANTIMICROBIALS USED ALONE

Based upon the concentration necessary to prevent growth of the yeast for 24 hr at 32°C or after subculture in fresh juice, sodium metabisulfite (sulfite) was found to be the most effective ($P < 0.05$) compound tested in both apple and grape juice (Tables 2, 3, 4, 5). The next most effective compounds in order of activity were butylated hydroxyanisole (BHA) and potassium sorbate. Tertiary butylhydroquinone (TBHQ) was not found to be a consistently effective inhibitor and was therefore not included in any statistical comparisons. The yeast strains were also found to react differently to the antimicrobials (Tables 3 and 4). Saccharomyces cerevisiae NRRL Y567 was shown to be more susceptible ($P < 0.05$) than S. cerevisiae. The difference between the two strains was most probably due to the greater susceptibility of S. cerevisiae NRRL Y567 to potassium sorbate in the inhibition study (Table 2) and S. cerevisiae to sulfite in the lethality study (Table 3). Potassium sorbate was not included in the lethality study because none of the concentrations tested were lethal (up to 600 µg/ml).

A maximum of 80 µg/ml of sodium metabisulfite was required to inhibit the growth of S. cerevisiae for 24 hr at 32°C in both apple and grape juice (Table 2). Saccharomyces cerevisiae NRRL Y567

TABLE 2. Maximum Inhibitory Concentrations ($\mu\text{g/ml}$) for Antimicrobials Against Saccharomyces cerevisiae and Saccharomyces cerevisiae NRRL Y567 in Apple Juice and Grape Juice After 24 Hours at 32°C

	Apple Juice	Grape Juice
Sulfite		
<u>S. cerevisiae</u>	80	80
<u>S. cerevisiae</u> NRRL Y567	100	100
Sorbate		
<u>S. cerevisiae</u>	500	600
<u>S. cerevisiae</u> NRRL Y567	400	400
BHA		
<u>S. cerevisiae</u>	160	140
<u>S. cerevisiae</u> NRRL Y567	120	160
TBHQ		
<u>S. cerevisiae</u>	600	900
<u>S. cerevisiae</u> NRRL Y567	600	1000

TABLE 3. Analysis of Variance of the Concentrations of Sodium Metabisulfite, Butylated Hydroxyanisole, or Potassium Sorbate Necessary to Inhibit the Growth of *Saccharomyces cerevisiae* and *Saccharomyces cerevisiae* NRRL Y567 in Apple or Grape Juice After 24 Hours at 32°C

Source	df	Mean Square	F Value
Strain (S)	1	55032.08	39.08*
Replication (R)	7	1777.31	1.26 ^{ns}
Juice (J)	1	5473.45	3.89 ^{ns}
Compound (C)	2	1883247.51	668.74*
S x J	1	1026.51	0.73 ^{ns}
S x C	2	70596.70	50.14*
J x C	2	3823.70	2.72 ^{ns}
R x S	7	352.41	0.25 ^{ns}
R x C	14	1927.04	1.37 ^{ns}
R x J	7	1265.57	0.90 ^{ns}
Error	49	1408.06	

* $P < 0.05$

^{ns} $P < 0.05$.

TABLE 4. Analysis of Variance of the Lethal Concentrations of Sodium Metabisulfite and Butylated Hydroxyanisole Necessary to Inhibit the Growth of Saccharomyces cerevisiae and Saccharomyces cerevisiae NRRL Y567 in Apple or Grape Juice After Subculture in Fresh Juice for 24 Hours at 32°C

Source	df	Mean Square	F Value
Strain (S)	1	6814.35	19.72*
Replication (R)	7	509.66	1.25 ^{ns}
Juice (J)	1	46.89	.04 ^{ns}
Compound (C)	1	22527.89	56.95*
S x J	1	2629.58	7.61*
S x C	1	925.64	2.68 ^{ns}
J x C	1	1126.61	3.26 ^{ns}
R x S	7	294.88	0.85 ^{ns}
R x C	7	166.50	0.48 ^{ns}
R x J	7	239.58	0.69 ^{ns}
Error	16	345.54	

* P<0.05

^{ns}

P<0.05.

TABLE 5. Maximum Lethal Concentrations ($\mu\text{g/ml}$) for the Antimicrobials Against Saccharomyces cerevisiae and Saccharomyces cerevisiae NRRL Y567 in Apple Juice and Grape Juice After 24 Hours Subculture at 32°C

	Apple Juice	Grape Juice
Sulfite		
<u>S. cerevisiae</u>	80	80
<u>S. cerevisiae</u> NRRL Y567	120	120
BHA		
<u>S. cerevisiae</u>	160	160
<u>S. cerevisiae</u> NRRL Y567	160	160

was slightly more resistant requiring 100 $\mu\text{g/ml}$ in both juices for inhibition of growth. The concentrations of sodium metabisulfite which prevented growth after subculture, and were therefore considered lethal to the microorganisms, were 80 $\mu\text{g/ml}$ for S. cerevisiae and 120 $\mu\text{g/ml}$ for S. cerevisiae NRRL Y567 in both juices (Table 5). The findings in the present study were in close agreement with previously published studies on sulfite. For example, Lueck (1980) reported that, at pH 4, 80-160 $\mu\text{g/ml}$ of sodium sulfite (50% sulfur dioxide) was required to inhibit the growth of S. cerevisiae. Splittstoesser and Mattick (1981) showed that 200 ppm ($\mu\text{g/ml}$) of sulfur dioxide completely inhibited the fermentation of grape juice by S. cerevisiae. Somewhat lower concentrations of sulfurous acid (0.1-20.2 $\mu\text{g/ml}$) were reported to be inhibitory to S. cerevisiae by Ough (1983). As reported in the "Review of Literature," King et al. (1981) stated that sulfurous acid was the most active form of sulfur dioxide, against yeast, and this could explain the lower inhibitory concentrations reported by Ough (1983).

The maximum concentration of BHA required to inhibit the growth of S. cerevisiae was 160 $\mu\text{g/ml}$ in apple juice and 140 $\mu\text{g/ml}$ in grape juice (Table 2). The lethal concentrations of BHA against S. cerevisiae was 160 $\mu\text{g/ml}$ in both juices (Table 5). Against S. cerevisiae NRRL Y567 the maximum inhibitory concentrations for BHA in apple juice was 120 $\mu\text{g/ml}$ and in grape juice was 160 $\mu\text{g/ml}$ (Table 2). The lethal concentrations for BHA in apple and grape juice were identical at 160 $\mu\text{g/ml}$ (Table 5). Eubanks and Beuchat (1982a; 1982b) found that

50-100 $\mu\text{g/ml}$ of BHA had little effect on the inhibition of S. cerevisiae, but at concentrations of 150 $\mu\text{g/ml}$ or greater a marked inhibition in the growth of this yeast occurred

The maximum inhibitory concentration of potassium sorbate against S. cerevisiae in apple juice was 500 $\mu\text{g/ml}$ (Table 2). The corresponding value for grape juice was 600 $\mu\text{g/ml}$. Saccharomyces cerevisiae NRRL Y567 appeared to be more susceptible to sorbate, being inhibited by 400 $\mu\text{g/ml}$ in both juices. As was stated previously, none of the concentrations of sorbate tested (up to 600 $\mu\text{g/ml}$) were found to be lethal to either yeast tested. The values obtained for sorbate were in agreement with those reported by other researchers. For example, Splittstoesser (1982) found that S. cerevisiae Montrachet 522 grew in the presence of 250 $\mu\text{g/ml}$ sorbic acid and 10% ethanol, but not in the presence of 500 $\mu\text{g/ml}$ sorbic acid and 10% ethanol. Ferguson and Powrie (1957) reported that 200-400 $\mu\text{g/ml}$ sorbic acid prevented yeast fermentation in apple juice. In contrast, Lueck (1980) reported that 25 $\mu\text{g/ml}$ sorbic acid inhibited Saccharomyces species at pH 3.2-5.7. This rather wide range of inhibitory concentrations could be due to a difference in the resistance of different species or strains to sorbate.

Tertiary butylhydroquinone had the highest maximum inhibitory concentrations of all the compounds tested requiring 600 $\mu\text{g/ml}$ in apple juice and 900 $\mu\text{g/ml}$ for grape juice to prevent the growth of S. cerevisiae for 24 hr at 32°C (Table 2). Against S. cerevisiae NRRL Y567, 600 $\mu\text{g/ml}$ TBHQ was required to inhibit growth in apple

juice and 1000 $\mu\text{g/ml}$ was required for inhibition of this yeast in grape juice. As with sorbate, none of the concentrations of TBHQ tested (up to 1000 $\mu\text{g/ml}$) were found to be lethal to S. cerevisiae or S. cerevisiae NRRL Y567. The results of studies by Eubanks and Beuchat (1982a; 1982b) agree closely with our data on TBHQ. They found that up to 250 $\mu\text{g/ml}$ of TBHQ had no significant effect on a strain of S. cerevisiae grown in yeast extract-malt extract-peptone-glucose (YMPG) broth; however, growth of the organism was inhibited by concentrations of 500-750 $\mu\text{g/ml}$ TBHQ.

2. INHIBITION BY COMBINATIONS OF THE ANTIMICROBIALS

Following inhibition testing with the individual compounds, the same antimicrobials were tested in the juices in various combinations. The purpose of these studies was to determine if the compounds interacted in a synergistic, additive or antagonistic manner against the growth of the yeast. To evaluate the influence of these compounds on each other the data were evaluated by chi-square analysis and prediction equations formulated from the analysis (Appendix, pages 71-76). The prediction equations were then used to generate three dimensional plots for each antimicrobial combination used in apple or grape juice against S. cerevisiae and S. cerevisiae NRRL Y567.

The first combination tested included the two compounds which were found to be most effective when used alone, i.e., sodium metabisulfite (sulfite) and BHA. From the three dimensional graph generated with the prediction equation, it can be seen that sulfite

alone would inhibit growth of S. cerevisiae in apple juice at a concentration of 80 µg/ml (Figure 1). In contrast, BHA used at 160 µg/ml did not give 100% inhibition, but when used with 60 µg/ml sulfite, 100 µg/ml BHA caused inhibition of the yeast growth. Against S. cerevisiae NRRL Y567 in apple juice, 80 µg/ml sulfite alone or 140 µg/ml BHA were required to achieve 100% inhibition of growth (Figure 2). From the generated plot, it was determined that a combination of 60 µg/ml sulfite and 80 µg/ml BHA would also result in growth inhibition.

With a combination of sodium metabisulfite and BHA in grape juice, S. cerevisiae was found to be inhibited by 80 µg/ml sulfite (Figure 3). As in apple juice, S. cerevisiae was not inhibited by 160 µg/ml BHA alone but a combination of 60 µg/ml sulfite and 80 µg/ml BHA resulted in 100% inhibition. When used against S. cerevisiae NRRL Y567, sulfite at 100 µg/ml or BHA at 140 µg/ml would cause inhibition (Figure 4). BHA at a level of 60 µg/ml used with sulfite at 80 µg/ml would also be sufficient to inhibit growth of this organism. In these studies, both yeasts were susceptible to sulfite, but S. cerevisiae appeared to have a greater resistance to BHA.

Three dimensional plots could not be produced for the studies designed to determine the lethal concentrations of the combinations of sulfite and BHA, therefore it was necessary to examine the raw data. As before, the concentrations which inhibited growth of the yeast after an additional 24 hr subculture in fresh juice were considered to be lethal. In apple juice, 80 µg/ml sulfite or

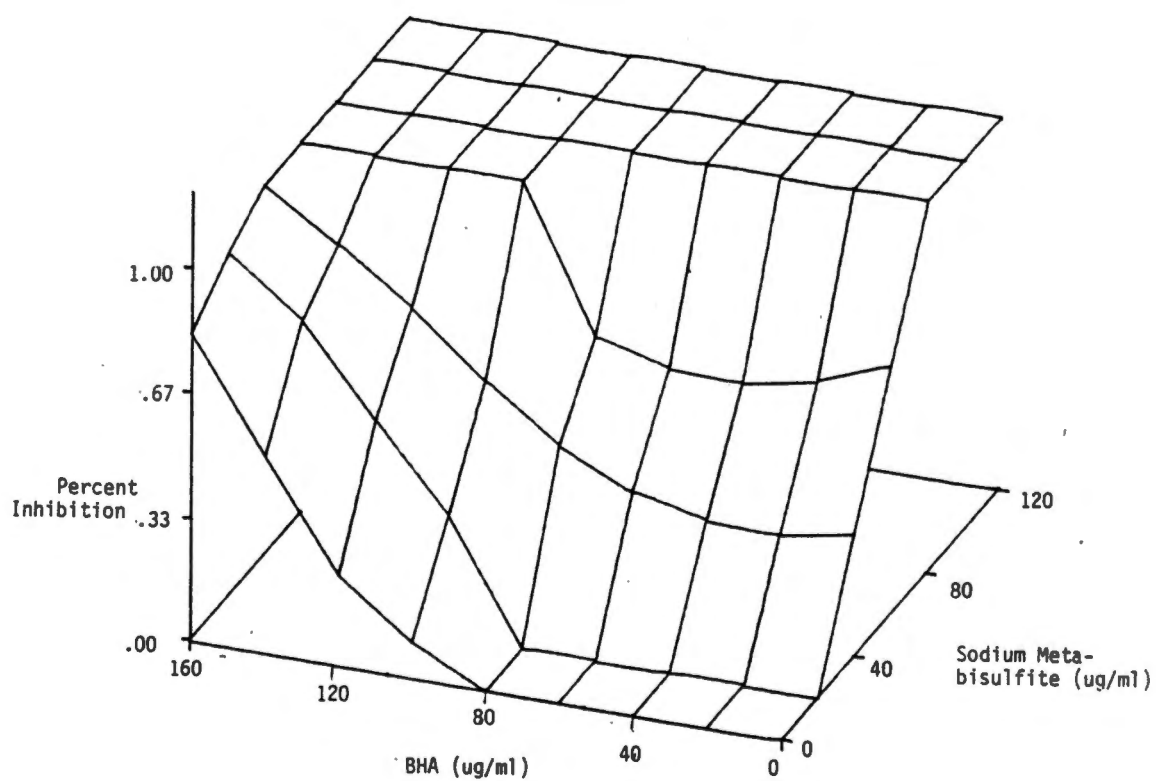


FIGURE 1. Inhibition of *Saccharomyces cerevisiae* in apple juice by combinations of butylated hydroxyanisole and sodium metabisulfite (24 hr at 32°C).

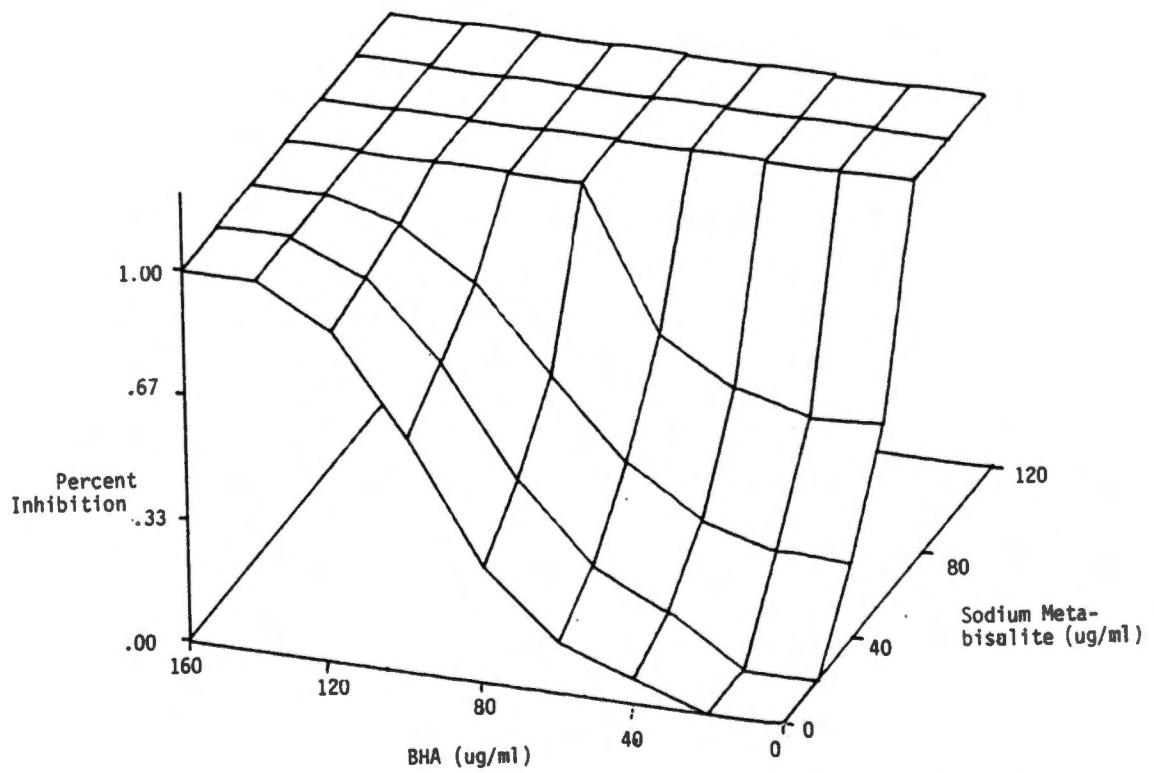


FIGURE 2. Inhibition of *Saccharomyces cerevisiae* NRRL Y567 in apple juice by combinations of butylated hydroxyanisole and sodium metabisulfite (24 hr at 32°C).

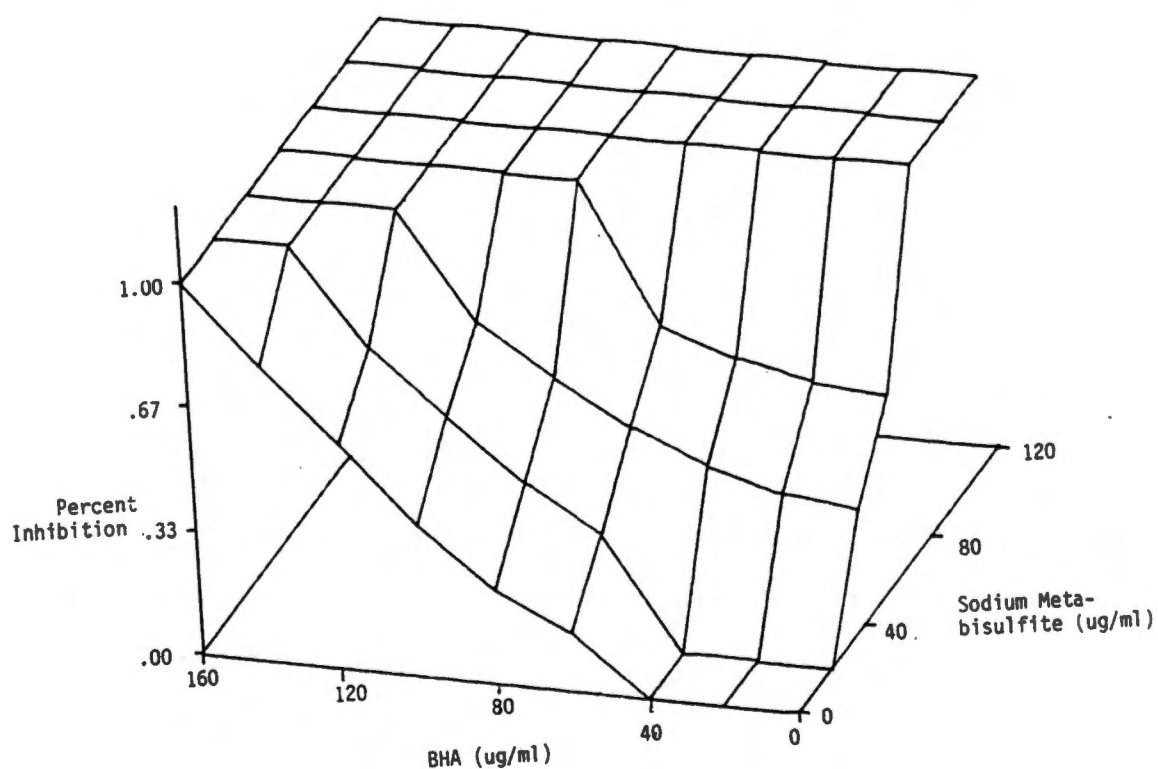


FIGURE 3. Inhibition of *Saccharomyces cerevisiae* in grape juice by combinations of butylated hydroxyanisole and sodium metabisulfite (24 hr at 32°C).

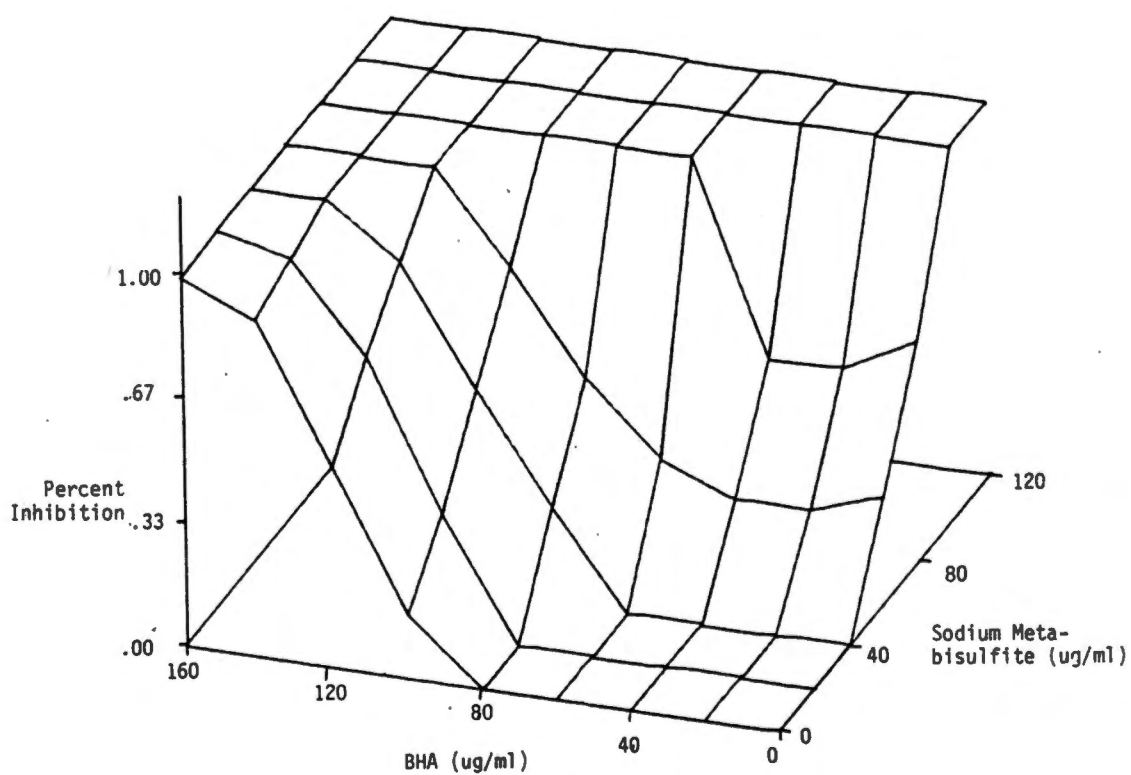


FIGURE 4. Inhibition of *Saccharomyces cerevisiae* NRRL Y567 in grape juice by combinations of butylated hydroxyanisole and sodium metabisulfite (24 hr at 32°C).

140 $\mu\text{g/ml}$ BHA when used alone were considered to be lethal to S. cerevisiae (Table 6). Concentrations of 120 $\mu\text{g/ml}$ sulfite or 160 $\mu\text{g/ml}$ BHA alone caused inhibition after 24 hr subculture of S. cerevisiae NRRL Y567 in apple juice (Table 7). When these antimicrobials were used in grape juice, inhibition occurred with S. cerevisiae at either 80 $\mu\text{g/ml}$ sulfite or 120 $\mu\text{g/ml}$ BHA (Table 8). Growth of S. cerevisiae NRRL Y567 was inhibited in grape juice after 24 hr subculture by 120 $\mu\text{g/ml}$ sulfite or 160 $\mu\text{g/ml}$ BHA used alone (Table 9). When increasing concentrations of BHA were combined with sulfite, lower sulfite levels could be used to obtain the same degree of inhibition of the yeast in all tests.

Because sodium metabisulfite and BHA are fairly reactive compounds (Ough, 1983; Rico-Munoz and Davidson, 1983), it might be expected that they would interact and their effectiveness would be reduced. Statistical analysis, however, revealed that the linear interaction of the compounds as it influenced the percentage inhibition was not significant ($P>0.05$), indicating additive but not antagonistic behavior.

From the prediction equation (Appendix, p. 73), it was determined that potassium sorbate at 600 $\mu\text{g/ml}$ or BHA at 140 $\mu\text{g/ml}$ would not cause 100% growth inhibition of S. cerevisiae in apple juice (Figure 5). When used in combinations, however, 400 $\mu\text{g/ml}$ sorbate and 80 $\mu\text{g/ml}$ BHA inhibited growth. Against S. cerevisiae NRRL Y567 in apple juice, either 400 $\mu\text{g/ml}$ sorbate or 120 $\mu\text{g/ml}$ BHA alone inhibited growth but the same compounds at 300 $\mu\text{g/ml}$ and 80 $\mu\text{g/ml}$ respectively, could also cause total inhibition (Figure 6).

TABLE 6. Growth of *Saccharomyces cerevisiae* in Tubes of Antimicrobial-Free Apple Juice After 24 Hours at 32°C Following Exposure to Combinations of Sodium Metabisulfite and Butylated Hydroxyanisole for 24 Hours at 32°C

BHA ($\mu\text{g/ml}$)	Sulfite ($\mu\text{g/ml}$)						
	0	20	40	60	80	100	120
0	-	-	1/1	1/3	0/2	0/2	0/2
20	-	-	1/1	1/3	0/2	0/2	0/2
40	-	-	0/1	0/2	0/2	0/2	0/2
60	-	-	0/1	0/3	0/2	0/2	0/2
80	-	-	1/2	2/4	0/2	0/2	0/2
100	-	-	0/2	1/4	0/2	0/2	0/2
120	0/1 ^a	1/2	0/3	1/4	0/2	0/2	0/2
140	1/3	1/3	1/4	1/4	0/2	0/2	0/2
160	2/4	1/4	0/3	0/3	0/2	0/2	0/2

^aNumber of tubes demonstrating growth after subculture/number of tubes demonstrating no growth in inhibitory testing.

TABLE 7. Growth of *Saccharomyces cerevisiae* NRRL Y567 in Tubes of Antimicrobial-Free Apple Juice After 24 Hours at 32°C Following Exposure to Combinations of Sodium Metabisulfite and Butylated Hydroxyanisole for 24 Hours at 32°C

BHA ($\mu\text{g/ml}$)	Sulfite ($\mu\text{g/ml}$)						
	0	20	40	60	80	100	120
0	-	-	0/1	2/2	4/5	3/4	0/1
20	-	-	1/1	2/2	3/5	3/3	0/0
40	-	-	-	2/2	4/5	2/3	0/0
60	-	-	-	3/3	3/5	2/2	0/0
80	-	-	1/2	3/5	2/2	0/0	0/0
100	3/3 ^a	3/3	3/3	3/3	0/2	0/0	0/0
120	1/1	3/5	3/5	1/3	0/2	0/0	0/0
140	4/5	2/5	1/3	0/0	0/0	0/0	0/0
160	0/2	0/2	0/0	0/0	0/0	0/0	0/0

^aNumber of tubes demonstrating growth after subculture/number of tubes demonstrating no growth in inhibitory testing.

TABLE 8. Growth of *Saccharomyces cerevisiae* in Tubes of Antimicrobial-Free Grape Juice After 24 Hours at 32°C Following Exposure to Combinations of Sodium Metabisulfite and Butylated Hydroxyanisole for 24 Hours at 32°C

BHA ($\mu\text{g/ml}$)	Sulfite ($\mu\text{g/ml}$)						
	0	20	40	60	80	100	120
0	-	-	1/1	1/3	0/2	0/2	0/2
20	-	-	1/1	1/4	0/0	0/0	0/0
40	-	-	1/2	1/2	0/0	0/0	0/0
60	-	-	1/1	1/4	0/0	0/0	0/0
80	1/1 ^a	1/1	2/3	0/4	0/0	0/0	0/0
100	1/2	1/2	1/4	0/2	0/0	0/0	0/0
120	1/3	2/4	0/1	0/1	0/0	0/0	0/0
140	2/4	1/4	0/1	0/1	0/0	0/0	0/0
160	0/1	0/1	0/1	0/1	0/0	0/0	0/0

^aNumber of tubes demonstrating growth after subculture/number of tubes demonstrating no growth in inhibitory testing.

TABLE 9. Growth of *Saccharomyces cerevisiae* NRRL Y567 in Tubes of Antimicrobial-Free Grape Juice After 24 Hours at 32°C Following Exposure to Combinations of Sodium Metabisulfite and Butylated Hydroxyanisole for 24 Hours at 32°C

BHA ($\mu\text{g/ml}$)	Sulfite ($\mu\text{g/ml}$)						
	0	20	40	60	80	100	120
0	-	-	-	1/1	1/1	3/4	0/4
20	-	-	-	1/1	3/3	3/4	0/4
40	-	-	-	1/1	4/4	0/4	0/0
60	-	-	-	1/1	3/4	0/4	0/0
80	-	-	-	1/1	2/4	0/0	0/0
100	-	-	1/1	0/4	0/2	0/0	0/0
120	2/2 ^a	3/3	1/4	0/0	0/0	0/0	0/0
140	2/3	1/4	0/0	0/0	0/0	0/0	0/0
160	0/4	1/4	0/0	0/0	0/0	0/0	0/0

^aNumber of tubes demonstrating growth after subculture/number of tubes demonstrating no growth in inhibitory testing.

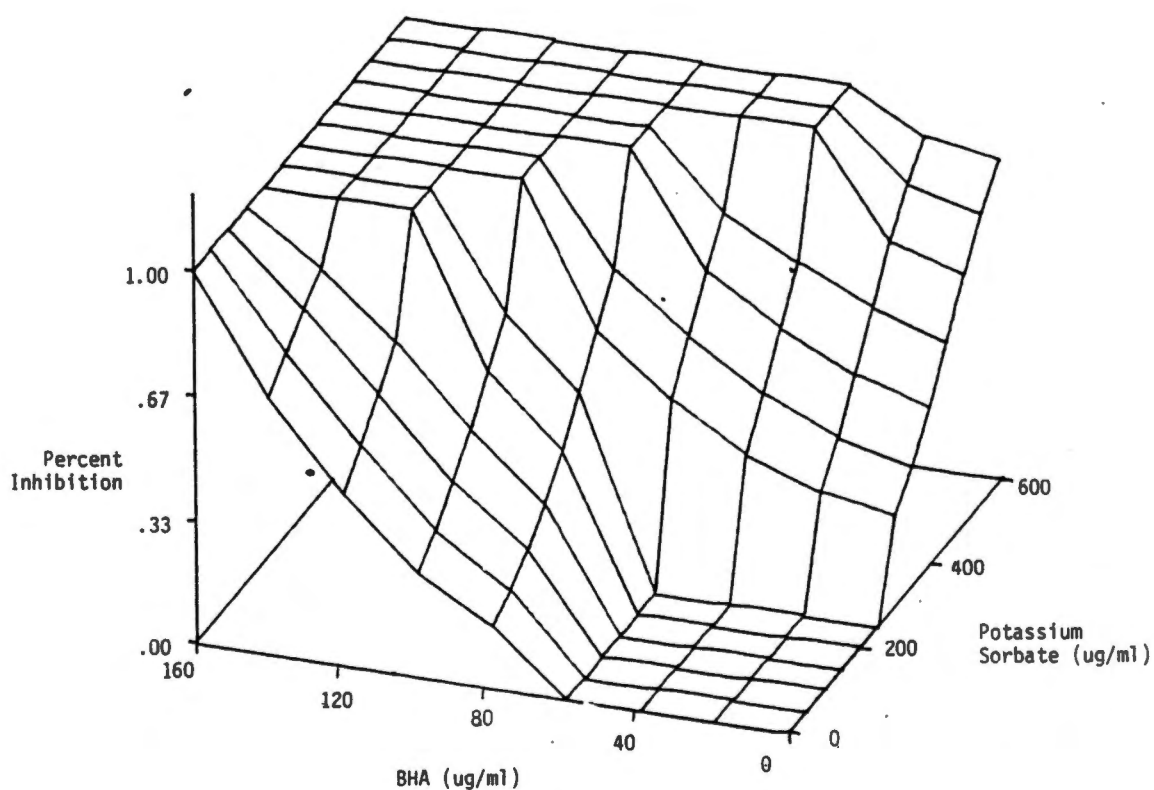


FIGURE 5. Inhibition of *Saccharomyces cerevisiae* in apple juice by combinations of butylated hydroxyanisole and potassium sorbate (24 hr at 32°C).

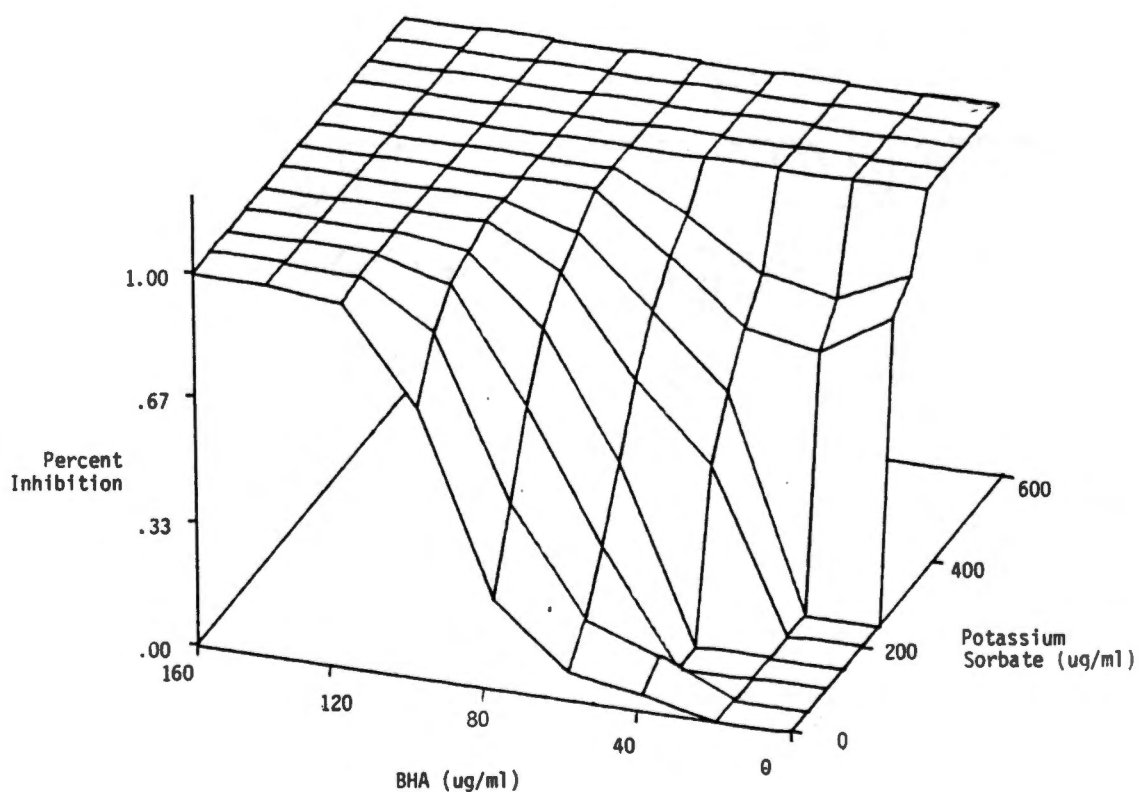


FIGURE 6. Inhibition of *Saccharomyces cerevisiae* NRRL Y567 in apple juice by combinations of butylated hydroxyanisole and potassium sorbate (24 hr at 32°C).

In grape juice, it was determined that S. cerevisiae was not completely inhibited in the presence of 550 µg/ml sorbate alone (Figure 7). In contrast, sorbate was shown to cause inhibition of S. cerevisiae growth at 200 µg/ml if combined with 100 µg/ml BHA. Against S. cerevisiae NRRL Y567, either 400 µg/ml sorbate or 140 µg/ml BHA totally inhibited yeast growth (Figure 8). When used in combination, 300 µg/ml sorbate used with 60 µg/ml BHA resulted in total inhibition. The difference in resistance of the two yeast strains to potassium sorbate was readily apparent with S. cerevisiae NRRL Y567 showing a lower resistance to the compound. As with BHA and sodium metabisulfite, the combined effects of sorbate and BHA were found to be additive since no significant interaction could be detected ($P>0.05$) between the two antimicrobials.

Previous studies carried out by Davidson et al. (1981), Lahellec et al. (1981) and Robach and Stateler (1980) found synergistic effects when BHA and sorbate were used against Staphylococcus aureus grown in different laboratory media. The difference between the present study and those in the literature on BHA/sorbate combinations could have been due to the variation in organisms, media, cultural conditions or data analysis since these factors have been shown to influence BHA activity in other studies (Branen et al., 1980).

From the plot of the sulfite and sorbate combinations (Figure 9), it can be determined that sulfite would inhibit S. cerevisiae in apple juice at 80 µg/ml. The sulfite appears to completely mask any inhibitory effect the sorbate may exert, i.e., none of the

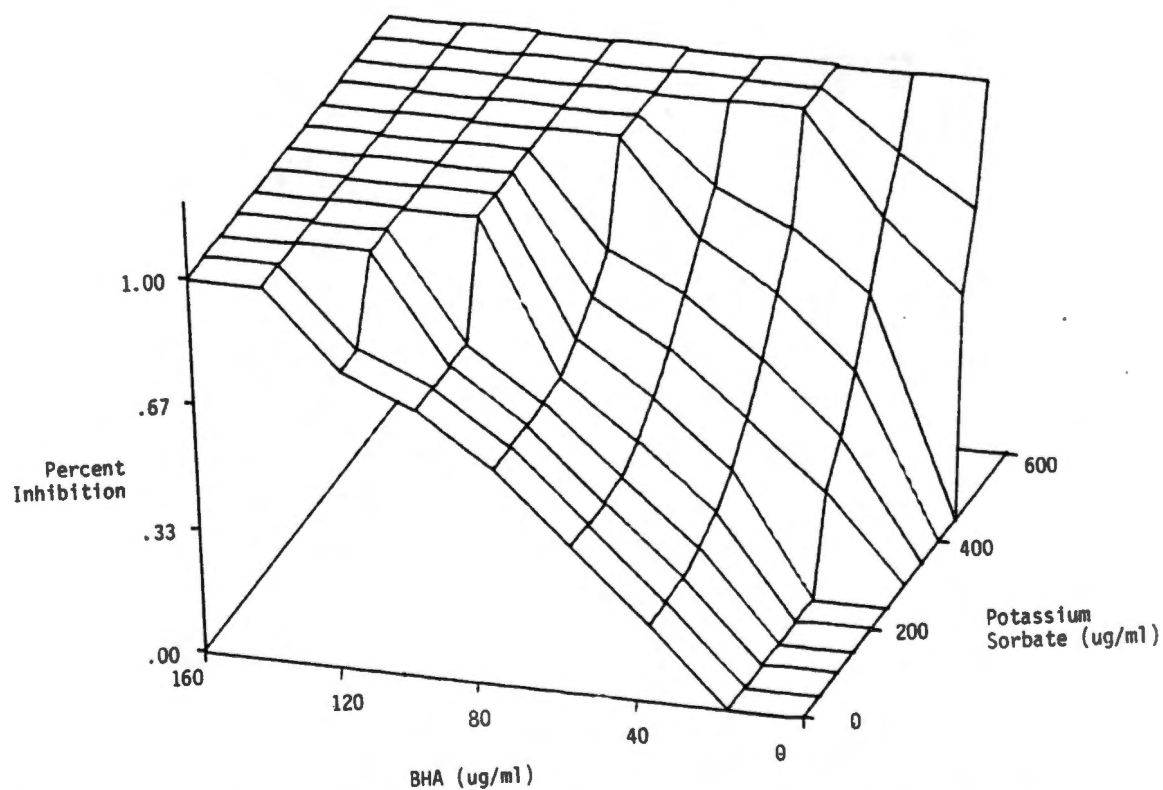


FIGURE 7. Inhibition of *Saccharomyces cerevisiae* in grape juice by combinations of butylated hydroxyanisole and potassium sorbate (24 hr at 32°C).

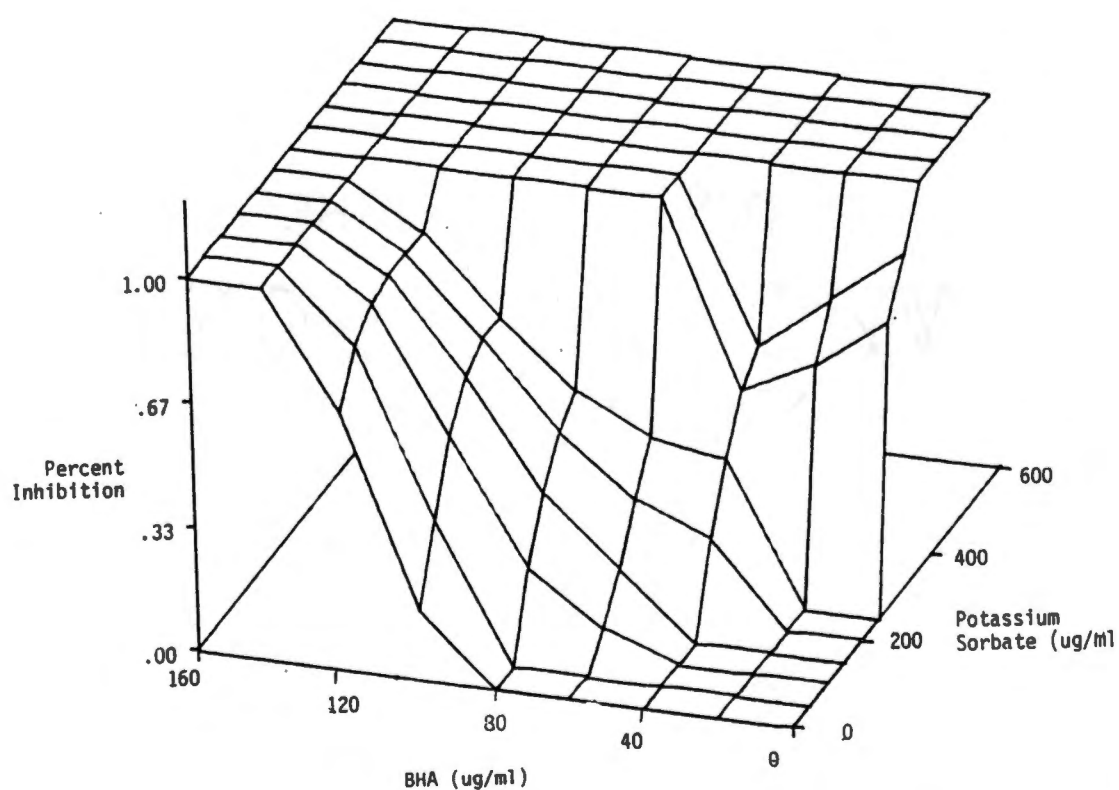


FIGURE 8. Inhibition of *Saccharomyces cerevisiae* NRRL Y567 in grape juice by combinations of butylated hydroxyanisole and potassium sorbate (24 hr at 32°C).

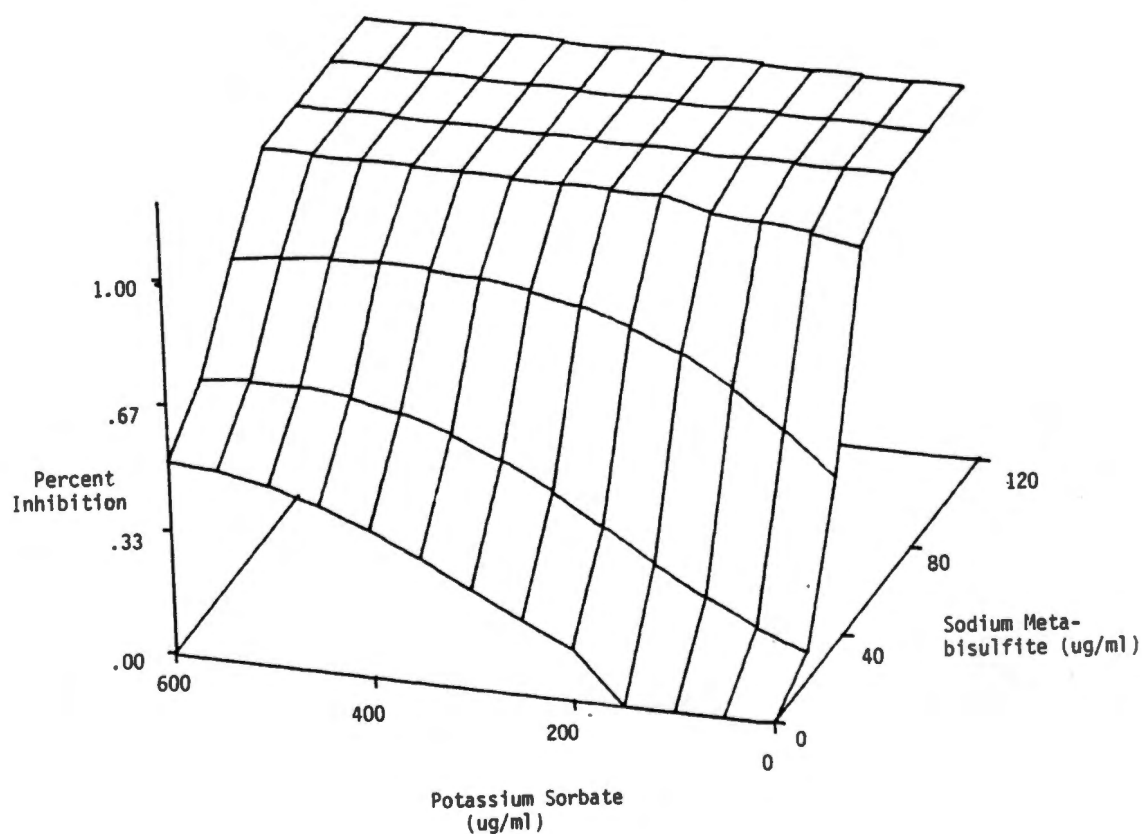


FIGURE 9. Inhibition of *Saccharomyces cerevisiae* in apple juice by combinations of potassium sorbate and sodium metabisulfite (24 hr at 32°C).

concentrations of sorbate appeared to inhibit the yeast until combined with 60 $\mu\text{g/ml}$ sulfite. Statistical analysis of these results indicated that no significant ($P>0.05$) interaction was occurring and that the compounds acted in an additive manner. Against S. cerevisiae NRRL Y567 in apple juice, 400 $\mu\text{g/ml}$ sorbate alone was needed for inhibition (Figure 10). Sulfite inhibited this yeast only at 120 $\mu\text{g/ml}$ or when combined with 100 $\mu\text{g/ml}$ sorbate. These results suggested an antagonistic interaction between sulfite and sorbate.

In grape juice, the generated data showed that S. cerevisiae was totally inhibited by 100 $\mu\text{g/ml}$ sulfite (Figure 11). Potassium sorbate caused 100% inhibition only at 600 $\mu\text{g/ml}$ or at 500 $\mu\text{g/ml}$ when combined with 40 $\mu\text{g/ml}$ sulfite. The most effective combination appeared to be 80 $\mu\text{g/ml}$ sulfite and 200 $\mu\text{g/ml}$ sorbate which caused inhibition of the organism. No interaction ($P>0.05$) was detected between sulfite and sorbate in this test so the effect of the anti-microbials was again considered to be additive.

If sorbate and sulfite were used against S. cerevisiae NRRL Y567 in grape juice, 300 $\mu\text{g/ml}$ sorbate alone would inhibit the organism, but total inhibition was not demonstrated with sulfite until it was combined with 200 $\mu\text{g/ml}$ sorbate (Figure 12). An interaction ($P<0.05$) was found to exist between these two compounds when they were used in combination in grape juice against this organism. It appeared that the sorbate had an antagonistic influence on the sulfite causing a higher concentration of sulfite to be needed to prevent growth. This is the only case where significant interactions occurred between

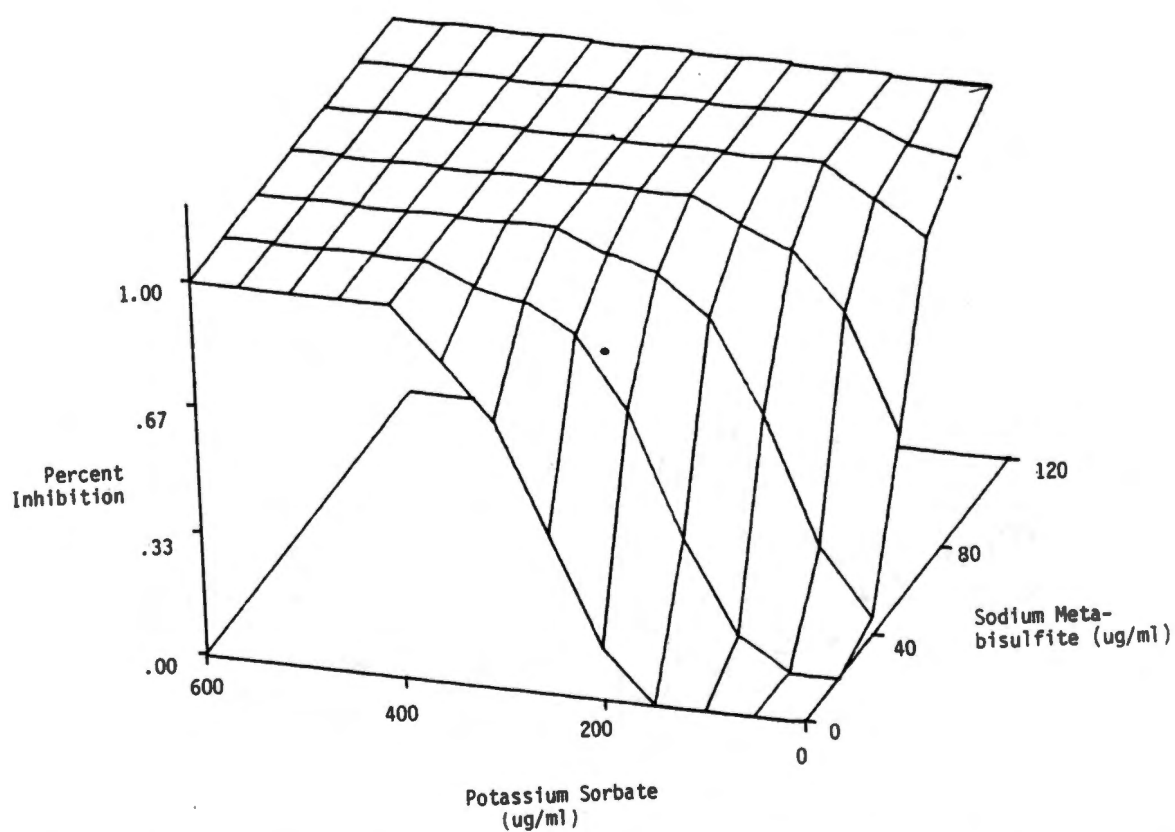


FIGURE 10. Inhibition of *Saccharomyces cerevisiae* NRRL Y567 in apple juice by combinations of potassium sorbate and sodium metabisulfite (24 hr at 32°C).

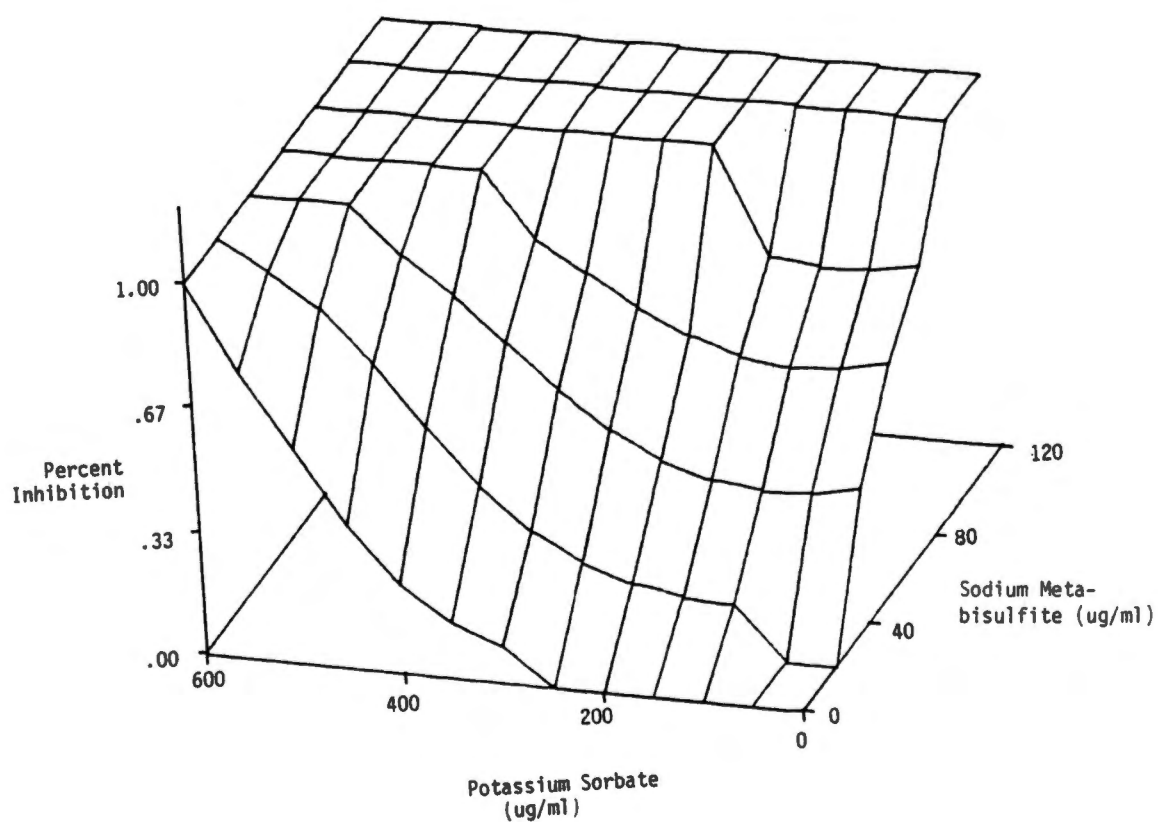


FIGURE 11. Inhibition of *Saccharomyces cerevisiae* in grape apple juice by combinations of potassium sorbate and sodium metabisulfite (24 hr at 32°C).

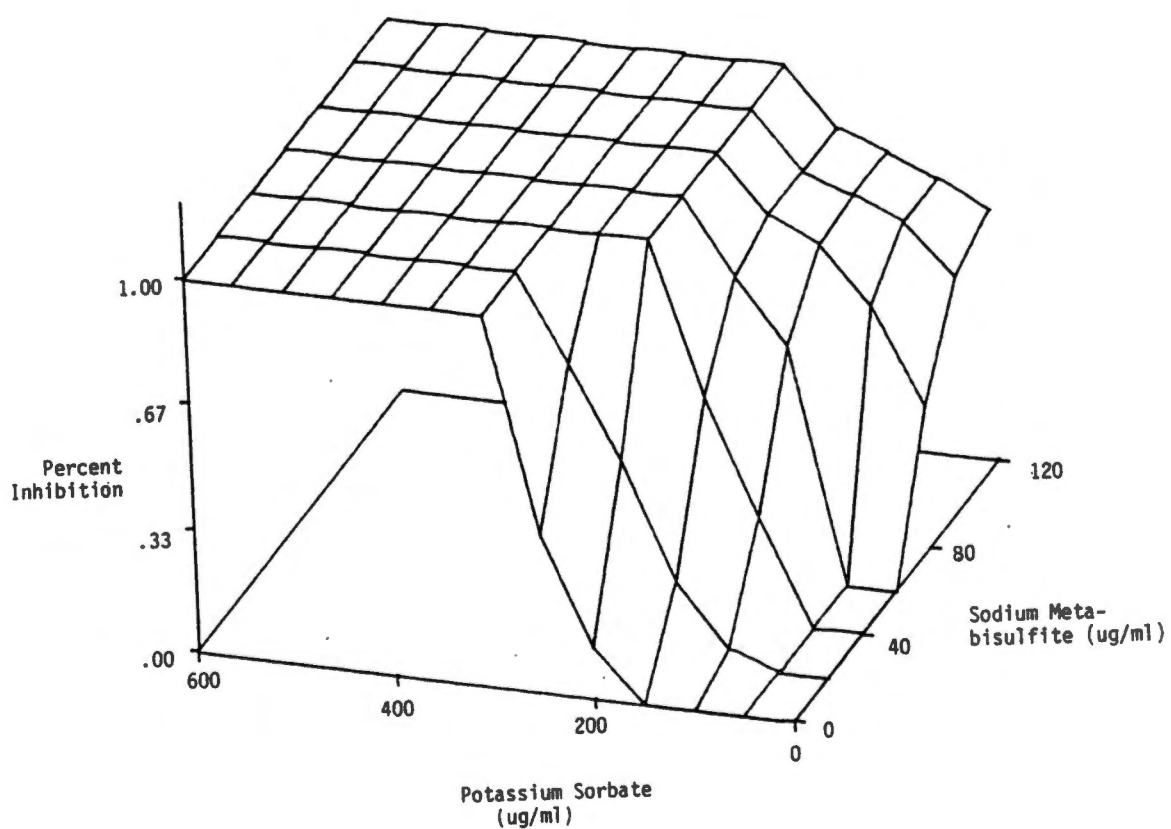


FIGURE 12. Inhibition of *Saccharomyces cerevisiae* NRRL Y567 in grape apple juice by combinations of potassium sorbate and sodium metabisulfite (24 hr at 32°C).

any of the combined antimicrobials. In both juices, S. cerevisiae showed a lower resistance to sulfite and a higher resistance to sorbate, while S. cerevisiae NRRL Y567 showed a greater susceptibility to sorbate and a lower susceptibility to sulfite.

Parish and Carroll (1984) have reported similar findings with combinations of sorbate and sulfur dioxide. They found that this antimicrobial combination did not display evidence of synergistic effects against S. cerevisiae Montrachet 522. These researchers also found that this combination appeared to provide less control than that of sulfur dioxide or sorbate used alone.

CHAPTER V

CONCLUSIONS

Sodium metabisulfite (sulfite), potassium sorbate (sorbate), butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ), were tested to determine their antifungal activity against two yeast strains: S. cerevisiae and S. cerevisiae NRRL Y567. The technique used to evaluate the antimicrobial activity of the compounds was modified from the tube dilution technique described by Barry (1976). Based upon the concentration necessary to prevent growth of the yeast, sulfite was found to be the most effective compound tested alone in both apple and grape juice. The next most effective compounds in order of their activity were butylated hydroxyanisole, potassium sorbate and tertiary butylhydroquinone. Only two of the compounds, sulfite and BHA, were lethal to the yeasts in the concentration range tested and again metabisulfite was most effective. A significant difference ($P < 0.05$) was found in the susceptibilities of the two yeasts to the compounds. This was probably caused by S. cerevisiae being more tolerant ($P < 0.05$) to potassium sorbate. In lethality testing, S. cerevisiae NRRL Y567 was significantly ($P < 0.05$) more tolerant to sodium metabisulfite than S. cerevisiae.

The importance of this research was that, while BHA used alone was not as effective as sulfite against S. cerevisiae and S. cerevisiae NRRL Y567, it was effective at relatively low concentrations.

These juices are one of the first food products in which BHA has been found to be an effective antimicrobial, most likely due to the absence of any appreciable lipid in the product. Since BHA is also an antioxidant, it could be effective as a dual function additive just as sulfite is an antimicrobial/antibrowning agent.

Following individual testing, the compounds, with the exception of TBHQ, were combined in the juices to determine if they acted synergistically, additively or antagonistically against the yeast. Sodium metabisulfite and BHA showed no significant interaction ($P > 0.05$) in either apple or grape juice against S. cerevisiae or S. cerevisiae NRRL Y567 indicating that these antimicrobials had no detrimental or beneficial influence on each other. The BHA and potassium sorbate combinations also failed to show any interaction indicating simple additive inhibition. When sulfite and sorbate were combined for use in apple and grape juice against S. cerevisiae and in apple juice against S. cerevisiae NRRL Y567, the combined effects were again found to be additive. In contrast, the activity of sulfite and sorbate in grape juice against S. cerevisiae NRRL Y567 was found to show a significant ($P < 0.05$) interaction. This interaction appeared to be antagonistic since a higher concentration of sulfite was needed in this study to inhibit yeast growth compared to the other studies using sulfite and sorbate.

Since combinations of antimicrobials were generally found to be additive in antimicrobial effectiveness, combining the antimicrobials would allow for a reduction in sulfite concentration but

no significant gain in antimicrobial activity would occur. If the primary goal was a reduction in sulfite concentration, then the use of the combinations would be acceptable.

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APPENDIX

PREDICTION EQUATIONS FOR THREE DIMENSIONAL GRAPHS

TABLE A1. Equation for the Percentage Inhibition of *S. cerevisiae* Using Butylated Hydroxyanisole and Sodium Metabisulfite in Apple Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	2.10	.1471	-4.9691
BHA (A)	1	0.01	.9069	.00522943
Sulfite (C)	1	1.81	.1782	.130257
A*A	1	1.82	.1771	.00022233
C*C	1	0.91	.3405	-.00059643
A*C	1	0.49	.4859	-.00043552

$$\text{Equation: } Y = -4.9691 + .00522943A + .130257C + .00022233A^2 - .00059643C^2 - .00043552(A*C)$$

TABLE A2. Equation for Percentage Inhibition of *S. cerevisiae* NRRL Y567 Using Butylated Hydroxyanisole and Sodium Metabisulfite in Apple Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	2.55	.1105	-3.45882
BHA (A)	1	0.03	.8690	.00704326
Sulfite (C)	1	0.49	.4856	.0434968
A*A	1	2.03	.1541	.000329246
C*C	1	0.19	.6654	.00019613
A*C	1	0.24	.6235	-.00026997

$$\text{Equation: } Y = -3.5882 + .00704326A + .0434968C + .000329246A^2 + .00019613C^2 - .00026997(A*C)$$

TABLE A3. Equation for Percentage Inhibition of *S. cerevisiae* Using Butylated Hydroxyanisole and Sodium Metabisulfite in Grape Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	7.66	.0056	-2.74431
BHA (A)	1	0.18	.6681	.00906555
Sulfite (C)	1	2.21	.1374	.052668
A*A	1	1.37	.2415	.000142385
C*C	1	0.15	.7019	-.00011455
A*C	1	0.18	.6700	-.00015308

$$\text{Equation: } Y = -2.74431 + .00936555A + .052668C + .000142385A^2 - .00011455C^2 - .00015308(A*C)$$

TABLE A4. Equation for Percentage Inhibition of *S. cerevisiae* NRRL Y567 Using Butylated Hydroxyanisole and Sodium Metabisulfite in Grape Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	0.37	.5411	-4.45831
BHA (A)	1	0.04	.8390	-.0198876
Sulfite (C)	1	0.10	.7494	.0542158
A*A	1	1.72	.1896	.000485498
C*C	1	0.01	.9358	.000077046
A*C	1	0.01	.9086	-.0001305

$$\text{Equation: } Y = -4.45831 - .0198876A + .0542158C + .000485498A^2 + .000077046C^2 - .0001305(A*C)$$

TABLE A5. Equation for Percentage Inhibition of *S. cerevisiae* Using Butylated Hydroxyanisole and Potassium Sorbate in Apple Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	8.53	.0035	-2.67826
BHA (A)	1	0.00	.9459	-.00128296
Sorbate (B)	1	0.24	.6233	.00275331
A*A	1	3.37	.0665	.00018664
B*B	1	0.89	.3459	7.895E-06
A*B	1	0.24	.6229	.00002262

$$\text{Equation: } Y = -2.67826 - .00128296A + .00275331B + .000018664A^2 + 7.895E-06B^2 + -.00002262(A*B)$$

TABLE A6. Equation for Percentage Inhibition of *S. cerevisiae* NRRL Y567 Using Butylated Hydroxyanisole and Potassium Sorbate in Apple Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	0.07	.7869	-1.93564
BHA (A)	1	0.17	.6758	-.0666456
Sorbate (B)	1	0.17	.6835	.0137787
A*A	1	1.01	.3153	.000956706
B*B	1	0.08	.7775	-.00001082
A*B	1	0.03	.8737	.000058232

$$\text{Equation: } Y = -1.93564 - .0666456A + .0137787B + .000956706A^2 - .00001082B^2 + .000058232(A*B)$$

TABLE A7. Equation for Percentage Inhibition of *S. cerevisiae* Using Butylated Hydroxyanisole and Potassium Sorbate in Grape Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	20.24	.0001	-3.65136
BHA(A)	1	16.78	.0001	.06532
Sorbate (B)	1	0.02	.8866	.000723241
A*A	1	5.01	.0252	-.00019565
B*B	1	2.56	.1096	.000013871
A*B	1	2.19	.1389	-4.138E-05

$$\text{Equation: } Y = -3.65136 + .06532A + .000723241B - .00019565A^2 + .000013871B^2 - 4.138E-05(A*B)$$

TABLE A8. Equation for Percentage Inhibition of *S. cerevisiae* NRRL Y567 Using Butylated Hydroxyanisole and Potassium Sorbate in Grape Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	1.47	.2257	-9.13086
BHA (A)	1	0.15	.6943	.0446339
Sorbate (B)	1	1.83	.1760	.0491479
A*A	1	0.51	.4744	.00031841
B*B	1	1.48	.2239	-.00005066
A*B	1	1.03	.3090	-.00027755

$$\text{Equation: } Y = -9.13086 + .0446339A + .0491479B + .00031841A^2 - .00005066B^2 - .00027755(A*B)$$

TABLE A9. Equation for Percentage Inhibition of *S. cerevisiae* Using Potassium Sorbate and Sodium Metabisulfite in Apple Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	4.75	.0292	-4.1011
Sorbate (B)	1	3.35	.0673	.0136278
Sulfite (C)	1	0.60	.4395	.063057
B*B	1	1.74	.1866	-1.112E-05
C*C	1	0.60	.4403	.000792505
B*C	1	0.71	.3997	-9.781E-05

$$\text{Equation: } Y = -4.1011 + .0135278B + .053056C - 1.112E-05B^2 + .000792505C^2 - 9.781E-05(B*C)$$

TABLE A10. Equation for Percentage Inhibition of *S. cerevisiae* NRRL Y567 Using Potassium Sorbate and Sodium Metabisulfite in Apple Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	5.54	.0185	-11.1856
Sorbate (B)	1	5.43	.0198	.0594968
Sulfite (C)	1	1.75	.1864	.245572
B*B	1	4.02	.0451	-.00006374
C*C	1	0.32	.5706	-.00107547
B*C	1	1.02	.3127	-.00049701

$$\text{Equation: } Y = -11.1856 + .0594968B + .245572C - .00006374B^2 - .00107547C^2 - .00049701(B*C)$$

TABLE A11. Equation for the Percentage Inhibition of *S. cerevisiae* Using Potassium Sorbate and Sodium Metabisulfite in Grape Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	8.51	.0035	-2.96417
Sorbate (B)	1	0.25	.6147	-.00356682
sulfite (C)	1	6.53	.0106	.0714161
B*B	1	3.16	.0754	.000020289
C*C	1	2.01	.1565	-.00027632
B*C	1	0.00	.9999	1.076E-08

$$\text{Equation: } Y = -2.96417 - .00356682B + .0714161C - .000020289B^2 - .00027632C^2 + 1.076E-08(B*C)$$

TABLE A12. Equation for the Percentage Inhibition of *S. cerevisiae* NRRL Y567 Using Potassium Sorbate and Sodium Metabisulfite in Grape Juice

Effect	df	Chi-Sq.	Prob.	Est.
Intercept	1	9.58	.0020	-10.9238
Sorbate (B)	1	7.88	.0050	0.0576269
Sulfite (C)	1	7.49	.0062	0.196703
B*B	1	5.06	.0245	-6.052E-05
C*C	1	4.75	.0293	-.00082985
B*C	1	4.92	.0265	-.00042164

$$\text{Equation: } Y = -10.9238 + .0576269B + .196703C - 6.052E-05B^2 - .00082985C^2 - .00042164(B*C)$$

VITA

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