CONTROLLING SECONDARY FERMENTATION WITH NEW PRESERVATIVES

by

CONSTANTINE MENELAOS ATHANASSIADIS

A THESIS

submitted to

OREGON STATE COLLEGE

in partial fulfillment of
the requirements for the
degree of

MASTER OF SCIENCE

June 1955

APPROVED:

Associate Professor of Food Technology

In Charge of Major

Head of Department of Food Technology

Chairman of School Graduate Committee

Dean of Graduate School

Date thesis is presented May 11, 1955

Typed by Margaret Suri

ACKNOWLEDGMENT

The author wishes to extend his sincere appreciation to Dr. H. Y. Yang for suggesting the topic, guidance, and continuous encouragement during the course of this investigation; to Dr. Earl M. Litwiller for his review of the manuscript; and to Dr. Jerome C. R. Li for his aid in the statistical analyses.

Gratitude is extended also to other members of the staff of the Department of Food Technology, and to my colleagues for their helpful advice and criticisms while the work was in progress.

TABLE OF CONTENTS

LIST OF TABLES

CONTROLLING SECONDARY FERMENTATION WITH NEW PRESERVATIVES

INTRODUCTION

Wine production is of great importance in the economy of a number of European countries, as well as the United States. The following figures taken from the Statistical Yearbook of the United Nations (18,p.169), and the Yearbook of Food and Agricultural Statistics (17,pp.69-70), give an indication of the size of the wine industry (Table 1).

Table 1

Wine Production in Thousands of Metric Tons

Region	Year			
	1934-38 (yearly average)	1948-50	1951	1952
Europe	15,570	13,570	15,070	13,900
United States	503	823	1,118.6	818.2
Greece	375.9	386	349.6	342.4
World (U.S.S.R. excluded)	19,500	18,000	19,800	17,700

The Pacific Northwest produces small quantities of wines, primarily from fruits other than grapes, especially berries

and apples.

One of the main problems in wine making is secondary fermentation. The winemaker may control fermentation during the first stage of rapid yeast growth by the use of special yeast cultures and sulfur dioxide. However, some types of wines, where there is unfermented sugar left or added (i.e. light sweet, or semi-sweet wines), can subsequently easily be contaminated with yeasts. This may result in a secondary fermentation which produces gas, turbidity, and sediment (23,p.134), making the product completely unmarketable.

Heretofore, sulfur dioxide has been used exclusively as the wine preservative, though it possesses certain disadvantages. The most serious disadvantages associated with the use of sulfur dioxide are the development of undesirable flavor, its interference with proper aging, and to a lesser extent the formation of precipitates and turbidity.

The purpose of this investigation was to study the preservative action of several antibiotics and of vitamin K_5, in controlling secondary fermentation. These preservatives were obtained from commercial sources, and their protective action was compared with that of sulfur dioxide.

The demand for a new preservative for the wine

industry is great, and in 1947, the National Wine Association petitioned for a hearing to the Federal Security Administrator to provide a tolerance for monochloroacetic acid in wine (22,p.58). The hearing was denied on the grounds that monochloroacetic acid as an additive in foods has been outlawed by the Food and Drug Administration for being poisonous.

With the hope of finding a wine preservative more suitable than sulfur dioxide, investigations have been carried out to evaluate several of the antibiotics and vitamins as agents for preventing secondary fermentation.

REVIEW OF LITERATURE

The use of different chemical compounds for the preservation of wines has been under constant consideration. A number of chemicals (1,p.316), have been tried and abandoned for various reasons. Sulfurous acid or molecular sulfur dioxide has proven to be the only one of value, and is currently the only such chemical that is legally permitted to be used in wines.

The effect of sulfurous acid or molecular sulfur dioxide on wines has been extensively studied during the last fifty years (3,pp.170-174;23,pp.134-38,157 and 6,pp. 18-21). When free, sulfur dioxide has a powerful antiseptic action on most spoilage microorganisms of the wines, and is able to control the character of the fermentation. Sulfur dioxide has a high antioxidative power, it clarifies the wines, dissolves the tartrates, and extracts and fixes the color of red wines (1,pp.311-315). Along with these advantages when used with special care, it possesses a number of disadvantages when used in excessive amounts. As Amerine and Joslyn mention (1,pp.89-90), when excessive amounts of sulfur dioxide are used there is a detraction from the original flavor of the wine, and an interfering effect with the natural aging. When copper salts are present, undesirable turbidities and deposits tend to be

produced. If high concentrations of sulfur dioxide are used over an extended period of time, sulfates are formed in large quantity, and a definite action of sulfurous acid on both metal and cement tank surfaces is often encountered. Yang and Wiegand (23,p.134) mention the possibility of the yeast becoming resistant to sulfur dioxide.

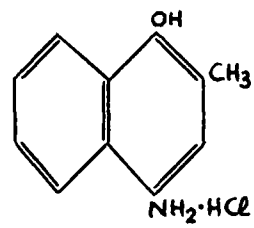
Because of the many disadvantages of sulfur dioxide, new compounds are being sought as wine preservatives, especially after the development of new chemicals and antibiotics. Walder (20,pp.100-103 and 21,pp.142-145), giving a historical review on the antibiotics and their application to food, gives methods of isolation and assay and discusses the experimental work which has been done in the field of food preservation. He points out the clear distinction between the addition of antibiotics to foods and the use of the usual chemical preservatives. He further points out the difference between an antibiotic substance and a preservative. By antibiotic he means a substance, of varied and complex formula, which is produced by a living organism, and antagonizes another living organism. By preservative he means a chemical compound, of simple structure (often inorganic) which is not usually found in nature, and which has bactericidal or fungicidal properties. In this work the word preservative refers to all

the additives that were used. In the fermentation industries and especially in wine making it is possible for organisms which may be desirable under normal conditions, to exert detrimental effects if they continue their function when it is no longer required. The reactions between the antibiotics and the microorganisms can be described as suppression, habituation, dependance and stimulation. In a recent article by Reed (11,p.25), it is reported that actually none of the work on the use of antibiotics in winemaking now in progress both in France and the United States has yet been published.

Penicillin, the well known antibiotic which was made commercially available immediately after World War II, has been comprehensively investigated, and a large number of applications have been found. Stranskov and Bockelmann (13,pp.1219-1223), working on the microbiological contamination of beer, used penicillin along with bacitracin, polymixin, and other antibiotics. Both penicillin and polymixin, at the concentrations used, showed a definitely promising effect by inhibiting and controlling bacterial infections of beer fermentations. Bacitracin and penicillin along with aureomycin and other antibiotics have been used at concentrations ranging from 0 to 2 ppm to test their preservative action on microorganisms isolated from meat (5,pp.165-166). Patulin, another antibiotic

used in this work, appears to be active against most microorganisms (16,p.350). Its use in the preservation of milk has been recently reported by two Japanese workers (7).

An analog of vitamin K, vitamin K_5 (2-methyl-4-amino-1-naphthol hydrochloride) with the following structure (19,p.77),



was found by Pratt et al. (9,pp.127-134) to exhibit a marked inhibitory activity toward a number of microorganisms, including Saccharomyces cerevisiae, the common brewer's yeast. Faggioli (4) mentions that the oxidation of vitamin K_5 by atmospheric oxygen causes a darkening effect. As Pratt et al. mention (10,p.128), the pinkish color that develops on standing does not alter the biological properties of the vitamin. The antibacterial activity of vitamin K_5 appears to depend on an intermediate product of oxidation by air which is formed immediately upon dissolving the compound (12,p.377).

MATERIALS AND METHODS

The experimental work in this investigation is divided into two main sections. The first is a study of the effects of certain additives on wine yeast (Saccharomyces ellipsoideus), grown on a synthetic culture medium, while the second section deals with the behavior of the most promising of these additives in relation to their inhibitory effect on secondary fermentation in wine.

Preparation of Synthetic Medium

The following liquid culture medium (6,p.18) was used throughout this work:

40 grams	Crystalline dextrose	(Baker Chemical Co.)
10 grams	Bactopeptone	(Difco Laboratories)
1000 ml.	Distilled water	

The pH of the medium was adjusted to 3.5 by the addition of a 10 percent solution of tartaric acid. Twenty milliliters of this solution per liter of medium gave the desired pH. This adjustment was carried out so that the medium would have a pH very similar to that of the wine.

Yeast Culture

The yeast culture (Saccharomyces ellipsoideus) that was used in this work, was obtained from the Enology Laboratory of the Food Technology Department of Oregon

State College. This culture is the one distributed by the above laboratory to the wine industries in Oregon, and is grown on dextrose-peptone-agar medium. The culture was activated by two successive transfers to liquid medium. At this stage of the work the condition of the culture was checked by using methylene blue staining (14,p.145), to differentiate dead and active yeast cells.

Preservatives Tested on Synthetic Medium

1. Penicillin "G" potassium U.S.P. (Nutritional Biochemicals Co.)

Units per mg.	1618
Expiration date	April 1, 1957

This potassium salt of penicillin is water soluble and is low in cost as compared with other forms of penicillin commercially produced. It is very stable in low temperature storage, nontoxic, but becomes inactivated in a short time at room temperature (16,pp.224-226). Four different concentrations of this antibiotic were tested: 1 ppm, 2 ppm, 3 ppm, and 5 ppm.

2. Bacitracin (Nutritional Biochemicals Co.)

Units per mg.	77.5
Expiration date	July 1955

Bacitracin appears to be a mixture of polypeptides. It is a water soluble, and very stable antibiotic at low pH. It

has a low toxicity, and resembles penicillin in its bacteriacidal spectrum (16,p.308). The concentrations used were the same as for penicillin.

3. Patulin (Nutritional Biochemicals Co.)

No specifications were given by the manufacturer. The concentrations used were the same as for penicillin.

4. Polymixin B sulfate (Nutritional Biochemicals Co.)

Units per mg.	6970
Expiration date	September 1956

Polymixin is a generic name indicating antibiotics obtained from strains of Bacillus polymyxa and the chemical composition is a basic polypeptide combined with a fatty acid ($C_8H_{17}COOH$). The polymixin B that was used in this experiment contained D-leucine, and phenylalanine. It is active against a number of microorganisms and has a low toxicity (16,pp.326-328). The concentrations used were the same as for penicillin.

5. Tetracycline Hydrochloride (Chas. Pfizer & Co.)

Units per mg.	934
Expiration date	July 1956

Tetracycline hydrochloride is a stable antibiotic and has a fairly low toxicity (16,pp.334-335). It was used at a concentration of 5 ppm.

6. Aureomycin hydrochloride (Lederle Laboratories)
(Chlorotetracycline hydrochloride)

No specifications were given by the manufacturer. Aureomycin hydrochloride is a rather unstable antibiotic though it is very effective on a great number of microorganisms. At low pH its stability is at a maximum. Its toxicity is fairly low. It was used in the same concentration as tetracycline hydrochloride.

7. Oxamycin (calcium salt)

8. Neomycin sulfate

9. Streptogramin

The above three antibiotics are recent developments of the Research Laboratories of Merck and Co. No specifications were given by the manufacturer and they were distributed to a number of research institutions for investigational purposes. They were used at a single concentration of 5 ppm.

10. Vitamin K_5 (Nutritional Biochemicals Co.)

No specifications were given by the manufacturer. Vitamin K_5 is a water soluble compound that was found to be very stable when protected from sunlight (9,p.128). It has a low toxicity and does not influence the flavor of any product to which it is added (10,p.323 and 4). The prepared solutions gave final concentrations of 100 ppm, 20 ppm, 10 ppm and 5 ppm (9,p.131).

11. Potassium metabisulfite, crystal (J. T. Baker Co.) This chemical was used as a comparative standard. The solutions prepared gave final concentrations in terms of sulfur dioxide of 300 ppm, 200 ppm, 150 ppm, 100 ppm and 75 ppm.

Methods of Testing Preservative Effect in Synthetic Medium

Round, screw-capped bottles of 115 ml. capacity were thoroughly cleaned and dried, and 49 ml. of the freshly prepared medium was introduced to each, with a 250 ml. burette. The bottles and their contents were then autoclaved at 15 lbs./sq. in. pressure for 15 minutes. For every concentration of the additive three replications were made. The stock culture and the stock solutions were introduced with sterile graduated pipettes after thorough mixing. Controls with no preservative added were also run in triplicate at the same time. After inoculation the bottles were placed in an incubator at 28° C. The production of alcohol was used as an index of the progress of fermentation, and was determined at the 7th and 15th day. If no growth appeared to have taken place at the 7th day, the first alcohol determination was delayed until the 15th day, and the second determination until the 30th day.

The alcohol was determined by a chemical method (8,pp.383-387) in preference to the official method because

it was considered to be more sensitive at these low alcohol concentrations. A slight modification was applied however, to permit more accurate results. This modification comprised an increase in the volume of the distilled samples from 10 ml. to 20 ml. This modification was employed throughout the entire investigation.

The stock solutions of the preservatives used were prepared so that when 0.5 ml. of these solutions were introduced into the bottles containing the medium and after the addition of 0.5 ml. of stock culture the total concentration of the preservative would be what was desired.

Preparation of Wine

Fresh grapes (Vitis vinifera var. Tokay) were obtained from Lodi, California. When received, they were inspected, and moldy berries and part of the stems were discarded. The grapes were placed in 10 pound cans and frozen at 0° F. They were stored at this temperature until they were used for the preparation of wine. Analysis of the grapes is shown in Table 2, page 14.

Table 2

Analysis of Grape Must

	pH	Total acids as tartaric, g./100 ml.	Total solids
(1)	3.25	0.503	19.9
(2)	3.30	0.495	19.8
(3)	3.20	0.503	19.4
Average	3.25	0.500	19.7

For winemaking a small quantity of the frozen grapes, approximately 5 pounds, were thawed and crushed by hand. Wine yeast culture (Saccharomyces ellipsoideus) was then introduced into the crushed grapes, and the preparation incubated at 28° C for 48 hours in a 3-liter glass container. After incubation the rapidly fermenting must was mixed with approximately 25 pounds of newly thawed and crushed grapes, placed in a five gallon glass jar, and again incubated at this temperature for 48 hours. The same procedure was repeated for a third time with approximately 125 pounds of grapes. The crushed grapes were allowed to ferment for a period of seven days. The fermentation was closely observed and when no further alcohol was produced the wine was separated by straining through cheese cloth. The young wine was placed in three 3-gallon jars and was stored at 32° F for a period of

three weeks. This cold storage has the advantage of complete inhibition of growth, as well as, the precipitation of the tartrates, resulting in a clearer wine. By storing the wine at this low temperature there were practically no possibilities for changes to take place during the period of four days which was necessary to prepare the four replications. The analyses of the wine before the three week storage and at the time of its utilization for the experimental work are shown in Table 3.

Table 3

Wine Analyses Before and After Storage

Determinations	Before storage	After Storage
Specific gravity at 20°/20° C	0.9940	0.9932
Percent alcohol by volume	11.6	11.6
Degrees Brix	-1.6	-1.8
Total acids as tartaric g./100 ml.	0.653	0.533
Volatile acids as acetic g./100 ml.	0.015	0.018
Reducing sugars g./100 ml.	0.115	0.115

The wine stored in three 3-gallon glass containers was siphoned to separate the sediment that was formed after the three week storage period. It was then thoroughly mixed, and samples were taken for the determinations reported in Table 3. Five percent by weight of dry

commercial sucrose was added to the wine prior to its experimental use to favor conditions for the initiation of a secondary fermentation.

Preservatives Used in Wine

Both polymixin and vitamin K_5 proved to have a definite effect on inhibiting fermentation in the synthetic medium, and were thus the preservatives used in this part of the work, while potassium metabisulfite was again used as the sulfur dioxide source for the standard reference. A preservative-free set of samples were used as a control. The characteristics of these preservatives were given in detail in section 3 of Materials and Methods. The stock solutions were prepared at such concentrations that when 1.0 ml. was added to each sample it brought the concentration of the preservative in the wine sample to the level shown in Table 4 page 17. These concentrations are within the effective range recommended by other investigators (5,p.165;10,pp.324-327;15,p.372 and 23,p.134).

Table 4

Concentrations of Preservatives in the Wine

Preservative	Concentrations in ppm		
Polymixin	2	3	4
Vitamin K_5	20	50	100
Sulfur dioxide	75	100	150

Fresh stock solutions were prepared every day, the previous ones being discarded after use to avoid possible change in activity due to storage.

Methods of Testing Preservative Effect in Wine

The procedure for testing in wine the three preservatives found to be most promising in a synthetic medium was as follows. Four replications were set up. Each replication consisted of 30 half-pint, screw-capped bottles, containing 149.0 ml. of wine. For each concentration of the preservatives tested three bottles were used plus three controls. The preservatives were added from stock solutions of such concentration that the addition of 1.0 ml. brought the final volume to 150.0 ml. at the desired concentration. All bottles were stored at room temperature. One bottle of each set was analyzed for alcohol content at 10, 20, and 30 day storage periods.

The alcohol was determined in all the samples according to the procedure described by the Association of Official Agricultural Chemists (2,p.170).

RESULTS AND DISCUSSION

Effect of Preservatives on Synthetic Medium

Results obtained from the experimental work on the synthetic medium are reported in Tables 5 and 6, pages 20 and 21. The alcohol production inhibition in percent was calculated according to the following equation:

$$q = \frac{a - b}{a} \times 100 ,$$

where "q" is alcohol inhibition in percent, "a" is the alcohol concentration of the control, and "b" is the alcohol concentration of the sample containing the preservative. This allows a more accurate comparison between the different preservatives and concentrations.

Table 5, page 20, gives the results of the preservatives that showed practically no inhibitory effect. In the case of Penicillin, Bacitracin and Patulin the medium was examined on the 7th and 15th days of incubation. The highest inhibitory effect after 7 days was obtained with Bacitracin in the four different levels used. This inhibitory effect amounted to less than 30 percent of the control and is considered unsatisfactory. The inhibition after a 15 day incubation period was insignificant.

In the case of Tetracycline hydrochloride Aureomycin hydrochloride, Oxamycin (Calcium salt), Neomycin sulfate, and streptogramin, the alcohol was determined after 15

Table 5

Alcohol Production, and Alcohol Production Inhibition, by Preservatives with Low Inhibitory Action*

Preservatives and concentrations		Alcohol production		Alcohol production inhibition in %	
		7 days	15 days	7 days	15 days
Penicillin	5ppm	1.33	1.74	15.3	3.3
	3ppm	1.42	1.74	9.6	3.3
	2ppm	1.42	1.76	9.6	2.2
	1ppm	1.47	1.74	6.4	3.3
Bacitracin	5ppm	1.14	1.77	27.4	1.7
	3ppm	1.25	1.77	20.4	1.7
	2ppm	1.24	1.75	21.0	2.8
	1ppm	1.35	1.76	14.0	2.2
Patulin	5ppm	1.44	1.79	8.3	0.6
	3ppm	1.41	1.80	10.2	0.0
	2ppm	1.42	1.80	9.6	0.0
	1ppm	1.36	1.80	13.4	0.0
Tetracycline HCl	5ppm		1.76		2.2
Aureomycin HCl	5ppm		1.77		1.7
Oxamycin	5ppm		1.77		1.7
Neomycin	5ppm		1.78		1.2
Streptogramin	5ppm		1.73		3.9
Control		1.57	1.80	0.0	0.0

*All figures are means of three determinations.

TABLE 6

ALCOHOL PRODUCTION, AND ALCOHOL PRODUCTION INHIBITION, BY PRESERVATIVES WITH HIGH INHIBITORY ACTION

Preservatives and Concentrations	Alcohol Production Determinations								Alcohol Production Inhibition in Percent	
	15 days				30 days				15 days	30 days
	1st	2nd	3rd	Average	1st	2nd	3rd	Average	Average	Average
Vitamin K_5										
100 ppm	0.00	0.43	0.00	0.14	0.00	0.43	0.00	0.14	92.2	92.2
50 ppm	0.96	1.12	1.26	1.11	0.98	1.18	1.25	1.14	38.3	36.7
20 ppm	1.45	1.70	1.27	1.47	1.48	1.72	1.32	1.51	18.3	16.1
10 ppm	1.57	1.71	1.78	1.69	1.71	1.78	1.80	1.76	6.1	2.2
5 ppm	1.78	1.80	1.80	1.79	1.79	1.80	1.80	1.80	0.6	0.0
Polymixin										
5 ppm	0.03	0.01	0.02	0.02	0.44	0.38	0.35	0.39	98.9	78.3
3 ppm	0.01	0.03	0.00	0.01	0.90	0.84	0.73	0.82	99.4	54.4
2 ppm	1.03	1.10	1.14	1.09	1.67	1.64	1.65	1.65	39.4	8.3
1 ppm	1.79	1.78	1.76	1.78	1.80	1.79	1.79	1.79	1.2	0.6
Sulfur dioxide										
300 ppm	0.00	0.00	0.00	0.00	0.00	0.24	0.23	0.16	100.0	91.1
200 ppm	0.00	0.00	0.00	0.00	0.43	0.34	0.39	0.39	100.0	78.3
150 ppm	0.65	0.52	0.68	0.62	1.21	1.27	1.23	1.24	65.6	31.1
100 ppm	1.20	1.21	1.11	1.17	1.65	1.59	1.61	1.62	35.0	10.0
75 ppm	1.54	1.48	1.44	1.49	1.76	1.70	1.80	1.75	17.8	2.8
Control	1.80	1.79	1.80	1.80	1.81	1.80	1.80	1.80	0.0	0.0

days of incubation, and the inhibition was also found to be insignificant, ranging from 1.2 to 3.9 percent.

Table 6 page 21 shows the alcohol production in the samples where polymixin, vitamin K_5, and sulfur dioxide were used. These three preservatives exerted a pronounced inhibitory effect on fermentation and are consequently reported in detail.

Polymixin. Polymixin had a high inhibitory effect on fermentation at both the 15 and 30 day incubation periods for the two highest concentrations of 3 and 5 ppm. This effect was more promounced on the 15 day storage as expected. The low concentrations had very little effect, which by the 30th day, for the 1 ppm concentration, was practically nil. As the period of storage increased the alcohol production inhibition showed a fairly pronounced decrease.

Vitamin K_5. Vitamin K_5 exerted an inhibitory effect that was fairly constant for the two periods of storage. The highest concentrations again gave the highest inhibitory effect on the alcohol production. At a concentration of 100 ppm, 92.2 percent inhibition was achieved and remained constant for the 15 and 30 day periods. The constancy of the effect of the three highest concentrations for the two determination periods is of great value because it keeps this effect at practically the same rate,

indicating a more lasting action.

Sulfur Dioxide. This preservative showed a distinct inhibitory effect as the concentration increased from 75 to 300 ppm. The two highest concentrations of 200 and 300 ppm resulted in no increase in alcohol production at the end of 15 days of incubation, showing a 100 percent inhibition. However, these two concentrations exhibited lower inhibitory effects of 78.3 percent and 91.1 percent respectively on the 30th day of incubation indicating that sulfur dioxide does not possess a lasting action. This is also indicated by the greater decreases in inhibitory power noted with the lower concentrations at the end of the 30 days period.

Effect of Preservatives on Wine

Results of the inhibitory effects of vitamin K_5, polymixin, and sulfur dioxide on the secondary fermentation of the wine used in this investigation are shown in Table 7 page 24.

The data collected were subjected to statistical analysis in order to determine whether the addition of these chemicals at the different concentrations and storage periods exerted any effect on secondary fermentation. Analysis of variance results are reported in Table 8 page 25, and from these results the following may be concluded:

Table 7

Means of Alcohol Production

Vitamins	10 days	20 days	30 days	Conc. mean	Treat. mean
Vitamin K_5					
" 100 ppm	11.63	11.63	11.63	11.63	
" 50 ppm	11.60	11.63	11.60	11.61	11.62
" 20 ppm	11.63	11.60	11.60	11.61	
Mean	11.62	11.62	11.61		
Polymixin					
" 4 ppm	12.45	13.68	14.54	13.55	
" 3 ppm	12.48	13.65	14.65	13.59	13.59
" 2 ppm	12.43	13.75	14.68	13.63	
Mean	12.45	13.69	14.63		
Sulfur dioxide					
" 150 ppm	11.65	11.80	13.73	12.39	
" 100 ppm	12.20	13.08	14.73	13.33	13.14
" 75 ppm	12.48	13.88	14.73	13.69	
Mean	12.11	12.92	14.39		
Control	12.65	13.85	14.80	13.77	13.77
Storage mean	12.12	12.85	13.67		

Table 8

Analysis of Variance

Percentage by Volume of Alcohol Production

Variation Source	Sum of squares	Degrees of freedom	Mean square	F
Treatments	87.7891	3	29.2630	6,361.52**
Vitamin K_5				
Concentrations	0.0023	2	0.0012	0.25
Storage	0.0006	2	0.0003	0.07
Conc. x storage	0.0027	4	0.0007	0.15
Polymixin				
Concentrations	0.0417	2	0.0209	4.52**
Storage	28.7817	2	14.3909	3,128.46**
Conc. x storage	0.0666	4	0.0167	3.61**
Sulfur dioxide				
Concentrations	10.8206	2	5.4103	1,176.15**
Storage	32.1706	2	16.0853	3,496.80**
Conc. x storage	2.0194	4	0.5049	109.42**
Control				
Storage	9.2867	2	4.6434	1,009.43**
Error	0.4014	87	0.0046	
Total	171.4120	119		

**Highly significant.

Effect of Preservatives. The preservatives are definitely effective in the inhibition of the secondary fermentation in the wine, regardless of storage periods and concentrations. Vitamin K_5 is most effective followed by sulfur dioxide, which is more effective than polymixin. These results are shown in Table 9.

Table 9

Effect of Preservatives on Wine

Treatments	Alcohol produced, %
Vitamin K_5	11.62
Polymixin	13.59
Sulfur dioxide	13.14
Control	13.77

L.S.D. 0.04 at the 0.01 percent level of probability.

Effect of Concentration. Regardless of storage period the effect of different concentrations of each preservative is as follows:

1. Vitamin K_5: The inhibitory effect of vitamin K_5 was constant for the three concentrations (i.e. 20, 50, and 100 ppm). This is illustrated by comparing the means of the different concentrations in Table 10 page 27.

Table 10

Effect of Concentration of K_5 on Secondary Fermentation in Wine

Concentrations	Alcohol produced, %
100 ppm	11.63
50 ppm	11.61
20 ppm	11.61

L.S.D. 0.07 at the 0.01 percent level of probability.

2. Polymixin: The inhibitory effect of polymixin was not the same for the three concentrations (i.e. 2, 3 and 4 ppm). A significant difference existed between the highest and the lowest concentrations used. Table 11 illustrates these results.

Table 11

Effect of Concentration of Polymixin on Secondary Fermentation in Wine

Concentrations	Alcohol Produced, %
4 ppm	13.55
3 ppm	13.59
2 ppm	13.63

L.S.D. 0.07 at the 0.01 percent level of probability.

3. Sulfur dioxide: The inhibitory effect of sulfur dioxide differed significantly according to concentration. The higher the concentration the greater was the inhibitory effect of this preservative. This is illustrated in Table 12.

Table 12

Effect of Concentrations of Sulfur Dioxide on Secondary Fermentation in Wine

Concentrations	Alcohol produced, %
150 ppm	12.39
100 ppm	13.33
75 ppm	13.69

L.S.D. 0.07 at the 0.01 percent level of probability.

Effect of Storage Period. The storage period exerted a highly significant effect on alcohol production. The longer the storage period the higher was the alcohol production. These results are shown in Table 13, page 29.

Table 13

Effect of Storage Period on Alcohol Production

(Means of All Treatments)

Storage period	Mean alcohol, %
10 days	12.12
20 days	12.85
30 days	13.67

L.S.D. 0.04 at the 0.01 percent level of probability.

1. Control: The effect of storage on the control produced highly significant results. By prolonging the storage period a higher concentration of alcohol was obtained. The results are illustrated in Table 14.

Table 14

Effect of Storage on Different Treatments

Storage periods	Alcohol, percent by volume Treatments			
	Vit. K_5	Polym.	SO_2	Control
0 days	11.60	11.60	11.60	11.60
10 days	11.62	12.45	12.11	12.65
20 days	11.62	13.69	12.92	13.85
30 days	11.61	14.63	14.39	14.80
L.S.D.*	0.07	0.07	0.07	0.13

*At the 0.01 percent level of probability.

2. Vitamin K_5: Storage did not exert any effect on alcohol production when vitamin K_5 was used. The inhibition was complete at all periods. The results are shown in Table 14.

3. Polymixin: The results obtained from this chemical are shown in Table 14 and indicate a highly significant effect of storage on the alcohol production which increased as the storage period increased.

4. Sulfur dioxide: Highly significant results were obtained as regards to storage periods with the preservative. The longer the storage period the greater was the alcohol produced. These results are shown in Table 14 page 29.

Interaction between Concentration of Preservative and Storage Period. In the case of vitamin K_5 there was no interaction between storage period and concentration. In the case of polymixin the 2 ppm concentration seemed to inhibit the most at the 10 day period, while the 3 ppm concentration seemed to inhibit the most at the 20 day period, and the 4 ppm seemed to exert the most inhibition at the 30 day period. Sulfur dioxide at the 150 ppm concentration inhibits growth at a much higher rate for the 10 and 20 day storage periods than the other two

concentrations.

A graphic representation of all the results on wine is shown in Figure 1.

Figure 1

Percent alcohol of different storage periods
30 DAYS
20 "
10 "
0 "
Alcohol percent by volume
15
14
13
12
11
Control
SQ 75 ppm
SQ 100 ppm
SQ 150 ppm
Polym. 2 ppm
Polym. 3 ppm
Polym. 4 ppm
Vit. K5 20 ppm
Vit. K5 50 ppm
Vit. K5 100 ppm

SUMMARY AND CONCLUSIONS

The control of secondary fermentation in wine with new preservatives was investigated. A synthetic medium inoculated with wine yeast (Saccharomyces ellipsoideus) was used in the preliminary stage of the work to evaluate eleven preservatives. These were considered promising by several investigators. The alcohol production was used as the index of the effectiveness of the compounds in controlling fermentation.

The second phase of the work comprised the addition of the most effective preservatives among those tried, to grape wine prepared especially for this purpose. Prior to the addition of the preservatives, five percent of commercial sucrose was added to the wine after completion of the fermentation. The inhibition of the increase in the alcohol concentration was used as an index of the effectiveness of the preservatives in controlling secondary fermentation. The results were analyzed by statistical methods to determine the effects of the different preservatives and concentrations on the alcohol production inhibition at the different storage periods. The results indicated the following conclusions:

1. The maximum percent of alcohol was produced when no preservative was added to either the synthetic medium

or the wine. The production of alcohol increased as the time of storage was prolonged.

2. On the synthetic medium, only three of the eleven preservatives investigated (i.e. penicillin, bacitracin, patulin, polymixin, tetracycline hydrochloride, aureomycin hydrochloride, oxamycin, neomycin, streptogramin, vitamin K_5, and sulfur dioxide) exhibited appreciable inhibitory effects. These were polymixin, vitamin K_5, and sulfur dioxide, and were applied to the experimental wine.

3. The reference preservative, sulfur dioxide showed a marked inhibition of secondary fermentation at the 150 ppm concentration, but its effect was not lasting, and after the 20 day storage period it was ineffective. The lower concentrations did not cause any marked effect even at the 10 day period.

4. Polymixin did not exhibit a great effect in controlling secondary fermentation. Even at the highest concentration applied (4 ppm), the alcohol production was fairly high. Little differences were shown in alcohol production due to the three concentrations used.

5. Vitamin K_5 at all the concentrations used (i.e. 20, 50, and 100 ppm) inhibited the alcohol production completely, showing great possibilities for controlling secondary fermentation in wine. The time of storage did

not have any influence on the action of vitamin K_5, indicating its lasting power.

BIBLIOGRAPHY

1. Amerine, M. A. and M. A. Joslyn. Table wines. Berkeley, University of California, 1951. 397p.

2. Association of official agricultural chemists. Official methods of analysis. 7th ed. Washington, Association of official agricultural chemists, 1950. 910p.

3. Cruess, W. V. The principles and practice of wine-making. 2d ed. New York, Avi, 1947. 476p.

4. Faggioli, Gino. Experiments with an analog of vitamin K as a food preservative. Igiene moderna 46:166-173. 1953. (Abstracted in Chemical abstracts 48(1):295b. 1954).

5. Goldberg, H. S., H. H. Weiser and F. E. Deatherage. Studies on meat. IV. The use of antibiotics in preservation of fresh beef. Food technology 7:165-166. April 1953.

6. Ingram, M. The germicidal effects of free and combined sulfur dioxide. Journal of the society of chemical industry 67:18-21. 1948.

7. Inomoto, Yoshiharu and Wataru Hashida. Application of several antibiotics to food industry. I. Preservation of cow milk. Journal of fermentation technology (Japan) 30:287-293. 1952. (Abstracted in Chemical abstracts 47(6):2894i. 1953).

8. Joslyn, Maynard A. Methods in food analysis applied to plant products. New York, Academic Press, 1950. 525p.

9. Pratt, Robertson et al. Vitamin K_5 as an antimicrobial medicament and preservative. Journal of the American pharmacological association 39:127-134. 1950.

10. Pratt, Robertson et al. Vitamin K_5 as an inhibitor of the growth of fungi and of fermentation by yeast. Proceedings ofthe national academy of sciences of the United States of America 34:323-328. 1948.

11. Reed, Gerald. A short review on recent work with antibiotics in fermentation. Americal journal of enology 5:23-25. 1954.

12. Shwartzman, Gregory. Antibacterial properties of 4-amino-2-methyl-1 naphthol hydrochloride (synkamin). Proceedings of the society for experimental biology and medicine 67:376-378. 1948.

13. Strandskov, F. B. and J. B. Bockelmann. Antibiotics as inhibitors of microbiological contamination in beer. Journal of agricultural and food chemistry 1:1219-1223. 1953.

14. Tanner, Fred Wilbur. The microbiology of foods. 2d ed. Champaign, Ill., Garrard Press, 1946. 1196p.

15. Tarr, H . L. A., John W. Boyd and H. M. Bissett. Experimental preservation of fish and beef with antibiotics. Journal of agricultural and food chemistry 2:372-375. 1954.

16. Underkofler, Leland A. and Richard J. Hickey. Industrial fermentations. Vol. 2. New York, Chemical publishing, 1954. 578p.

17. United Nations. Food and Agriculture organization. Yearbook of food and agricultural statistics. Production. Vol. 7(1), Rome, Italy, F.A.O.U.N., 1954. 334p.

18. United Nations. Statistical office. Department of economic affairs. Statistical yearbook 1953. 5th issue. New York, 1953. 578p.

19. Veldstra, H. and P. W. Wiardi. Water soluble antihemorrhagic substances. I. Recueil des travaux chimiques des PaysBas 62:75-84. 1943.

20. Walder, Winifred O. Antibiotics and food, part I. Food processing, packing, marketing 21:100-103. 1952.

21. Walder, Winifred O. Antibiotics and food, part II. Food processing, packing, marketing 21:142-145. 1952.

22. Wine firms denied preservative use. Food field reporter 15(8):58. April 14, 1947.

23. Yang, H . Y. and E. H. Wiegand. Possibilities of secondary fermentation in light sweet wines. The fruit products journal and American food manufacturer 28:134-138,157. 1949.