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INHIBITION OF Clostridium perfringens BY BUTYLATED HYDROXYANISOLE

K. J. KLINDWORTH, P. M. DAVIDSON, C. J. BREKKE and A. L. BRANEN

ABSTRACT

Several concentrations of butylated hyrdoxyanisole (BHA) were tested for effectiveness in inhibiting the growth of *C. perfringens*. Three strains of *C. perfringens* were inhibited by 150 ppm BHA in Fluid Thioglycollate Medium. BHA was equally effective as an autoclaved or filter-sterilized solution. Inhibition with 100 ppm was found to increase at the extremes of the pH range tested (5.5–8.5). With 100 and 200 ppm, BHA was found to be bactericidal to *C. perfringens* cells in a dilution buffer. BHA was found to have a synergistic inhibitory effect when used in conjunction with nitrite, sorbic acid, or esters of para-hydroxybenzoic acid (parabens). In the presence of a lipid and surfactant, the antimicrobial activity of BHA against *C. perfringens* was greatly reduced.

INTRODUCTION

THE RIGOROUS TESTING that is currently required by the Food and Drug Administration for the approval of new food additives has led to research by which new applications are being sought for currently approved additives. The antimicrobial activity of butylated hydroxyanisole (BHA), an antioxidant added to numerous food products, was first reported by Chang and Branen (1975). They found BHA to be inhibitory to *Aspergillus parasiticus* at 250 ppm, *Staphylococcus aureus* at 150 ppm, and *Escherichia coli* at 400 ppm. Ayaz (1975) studied the effects of BHA on *S. aureus* growth and enterotoxin production. He reported that 150 ppm BHA resulted in 93–97% inhibition of growth, and concentrations greater than 100 ppm inhibited toxin formation. Shih and Harris (1977) surveyed the antimicrobial activity of several antioxidants on selected bacterial species. They reported that BHA was more effective against *S. aureus* than against *E. coli*, confirming the work of Chang and Branen (1975). BHA was also found to inhibit *Vibrio parahemolyticus* at 50 ppm when used in Trypticase Soy Broth and at 400 ppm in a crab meat homogenate (Robach et al., 1977).

The purpose of this study was to determine the effects of BHA on the anaerobe *Clostridium perfringens*, a known food poisoning organism that is difficult to control due to its wide distribution and sporeforming ability. Several factors affecting BHA activity were investigated including heating of the medium, culture age, pH, and the presence of other antimicrobials and fat in the medium.

EXPERIMENTAL

Cultures

C. perfringens strains NCTC 10239, 8798 and 8239, were obtained from Dr. C.L. Duncan, University of Wisconsin, Madison. After revival in Cooked Meat Medium (Difco) at 37°C for 24 hr, the cultures were transferred to Fluid Thioglycollate Medium (FTM) Without Indicator-1350 (BBL, Cockeysville, MD), and grown at 45°C for 18 hr. From these cultures, inocula were prepared in FTM with incubation for 14 hr at 45°C. Thioglycollate broth reportedly supports only vegetative growth (Dework, 1972).

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Growth inhibition studies

Butylated hydroxyanisole (BHA) was obtained from Eastman Kodak Co., Kingsport, TN. The tablets were finely ground with a Sorvall Omnimixer (Dupont, Newtown, CT) and the powder was dissolved in 95% ethanol to give a 2% stock solution. Appropriate quantities of the 2% stock solution were pipetted into side-arm flasks containing 100 ml of FTM to give the desired final concentration, and the medium was autoclaved. The effect of the 95% ethanol, used to dissolve BHA, on the growth of *C. perfringens* NCTC 10239 was tested by use of a control in which 1 ml of 95% ethanol was added to FTM in a side-arm flask. This control was then autoclaved and inoculated along with the flasks containing BHA.

Following sterilization and cooling, 1 ml of a 14-hr seed culture of *C. perfringens* (containing approximately 1.5×10^9 organisms/ml) was added to each flask and the flasks were incubated in a water bath at 37° or 45°C. In early studies, the flasks were flushed with nitrogen gas after inoculation to remove the majority of the oxygen present. This was later found to have no effect on growth and the practice was discontinued. Culture growth was measured at specified intervals for 12 hr using a Klett-Summerson photoelectric colorimeter (Klett Manufacturing Co., Inc., NY) with a green filter (no. 54) of 520–580 nm wavelength. Percentage inhibition was calculated from these data using the formula:

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Control blank}) - (\text{BHA Treatment} - \text{BHA blank})}{(\text{Control} - \text{Control blank})} \times 100$$

Control and BHA blanks were the zero time readings for the individual flasks before any growth had occurred. When necessary the number of organisms was determined by use of Trypticase Sulfite Neomycin (TSN) agar (BBL) with incubation in GasPak anaerobic jars (BBL) at 45°C for 18 hr.

Studies were conducted to determine the effects of various factors on inhibition of *C. perfringens* by BHA:

Effect of autoclaving. A series of tests were conducted to determine whether any changes in BHA activity occurred as a result of autoclaving. Flasks were made with FTM in the usual manner. To half of the flasks, BHA was added and the media were autoclaved. The remaining flasks of media were first autoclaved, then BHA that had been filter-sterilized using a 0.45 μm membrane filter (Schleicher and Schuell, Keene, NH) was aseptically added. The flasks were then inoculated with 22 hr cultures of strains NCTC 10239 and NCTC 8239 and growth was monitored as before at 45°C.

Effect of pH. Fluid Thioglycollate Medium was prepared and adjusted with 1N NaOH or 1N HCl to give pH's of 5.5 through 8.5 at 0.5 ± 0.02 unit intervals. Stock BHA was added to give 0, 100 and 150 ppm solution and the medium was autoclaved. The pH was again measured, the flasks were inoculated with *C. perfringens* NCTC 10239 and growth monitored as described above.

Effect of culture age. Seed cultures of NCTC 10239 were grown in FTM at 45°C for 13, 15, 17, 19 and 21 hr. Flasks with BHA concentrations of 0, 100 and 150 ppm in FTM were inoculated with these cultures and growth was monitored with the Klett colorimeter. Plate counts on TSN were made to determine the initial level of cells in each flask.

Lethality rate of butylated hydroxyanisole

A dilution buffer was prepared using 0.1% peptone with 0.1% cysteine, 0.05% sodium thioglycollate, and 0.9% sodium chloride. The pH was adjusted to 7.0 ± 0.02 using 1N NaOH or 1N HCl.

Table 1--Percentage inhibition of three strains of C. perfringens by butylated hydroxyanisole[a]

ppm BHA	Percentage inhibition		
	NCTC 10239	NCTC 8798	NCTC 8239
0	0.0	0.0	0.0
50	0.0	9.3	8.3
100	2.9	34.5	29.5
150	100.0	100.0	100.0
200	100.0	100.0	100.0
400	100.0	100.0	100.0

[a] Tested in Fluid Thioglycollate Medium at 37°C, inhibition determined by Klett colorimeter readings after 12 hr growth. Percentage inhibition was calculated by:

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Control blank}) - (\text{Treatment} - \text{Treatment blank})}{(\text{Control} - \text{Control blank})} \times 100$$

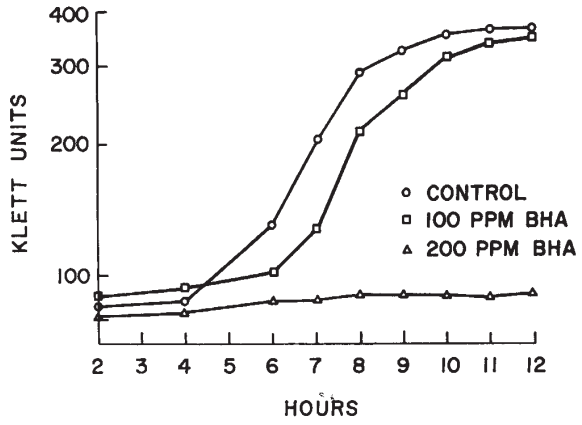

Fig. 1--Inhibition of C. perfringens NCTC 10239 by butylated hydroxyanisole in Fluid Thioglycollate Medium at 37°C.

Solutions were made containing 0, 50, 100 and 200 ppm BHA and were autoclaved. One milliliter of a seed culture of NCTC 10239 was added to each flask and, while being held at 37°C, a sample was withdrawn at 0.25, 5, 10, 20 and 30 min. The samples were serially diluted in 0.1% peptone dilution blanks, plated on TSN agar, and incubated anaerobically at 45°C for 24 hr.

Butylated hydroxyanisole and other food antimicrobials

Sodium nitrite, sorbic acid, and the esters of para-hydroxybenzoic acid (parabens) were tested to evaluate the possiblity of an inhibition of antimicrobial activity or a synergistic effect when these compounds are used in conjuntion with BHA. Initially, several concentrations of each of these additives were tested for antimicrobial activity against *C. perfringens* NCTC 10239. The two greatest concentrations in which growth occurred were used to test for antimicrobial interactions with BHA. In each case the antimicrobial solutions with BHA were autoclaved, inoculated and growth monitored using the Klett colorimeter as previously described.

Preliminary work with sodium nitrite showed 150 ppm to be completely inhibitory to *C perfringens* when added to FTM and autoclaved. Concentrations of 50 and 100 ppm sodium nitrite were used with 0, 50, 100 and 150 ppm BHA. Each sample was prepared by dissolving an appropriate amount of solid sodium nitrite in 100 ml FTM, adding stock BHA solutions and autoclaving. Filter-sterilized nitrite was also tested in the initial study. However, the concentrations that permitted growth of *C. perfringens* were so high that it was considered impractical to pursue testing since these levels (2000 ppm) greatly exceed the maximum allowed in foods.

Total inhibition of *C. perfringens* by sorbic acid alone occurred at a concentration of 0.1%. Concentrations of 0.05% and 0.075% Sorbistat® (Pfizer and Co. Inc., NY) were dissolved in 100 ml of FTM, and the pH was adjusted to 6.5 ± 0.02 with 1N NaOH and 1N HCl prior to the addition of BHA. Concentrations of BHA used were 0, 50, 100 and 150 ppm.

In initial work involving the parabens, methyl and propyl esters of parahydroxybenzoic acid in 2:1 and 3:1 ratios (Chichester and Tanner, 1968) were used. Solutions were made by dissolving appropriate quantities of the methyl ester (Pfaltz and Bauer, Inc., Stamford, CT) and the propyl ester (Sigma Chemical Co., St. Louis, MO) in 95% ethanol to give 5%, 7.5% and 10% w/v solutions. Both mixtures were tested at concentrations of 0.05%, 0.075% and 0.1% for their effect on *C. perfringens*. All concentrations were inhibitory except the 3:1 ratio at 0.05%. For work with BHA, 0.025% and 0.05% solutions of the 3:1 ratio were used. The pH was adjusted to 7.0 ± 0.02, and BHA at 0, 50, 100 and 150 ppm was added.

Effect of lipids on the antimicrobial activity of butylated hydroxyanisole

Trypticase Sulfite Neomycin (TSN) Agar served as the basal medium for this study. Corn oil (Mazola) was used to obtain lipid concentrations of 1, 2, 3, 4 and 5% (v/v). Polyoxyethylene sorbitan monooleate (Tween 80®, Atlas Powder Co., Wilmington, DE) was utilized at 0.5% to insure dispersion of the oil. This compound is an emulsifier with a high hydrophile-lipophile balance (HLB), suitable for oil-in-water emulsions (Griffin and Lynch, 1968). Each lipid concentration was tested with and without BHA at 200 ppm. The control (0% lipid) was TSN without any other ingredients. In addition, the effects of the other ingredients were tested by use of treatments with TSN and BHA only; TSN and Tween 80® only; and TSN with Tween 80® and BHA. The TSN agar was prepared according to the manufacturer's directions and quantities were dispensed into 200 ml prescription bottles. The quantities added were such that when the oil was added later, the final volume was 150 ml excluding the volume due to the emulsifier and BHA. The BHA was then added to the appropriate bottles and all the bottles were autoclaved for 15 min at 121°C. After autoclaving, the bottles were held at 52°C and the emulsifier and corn oil were added aseptically. Pour plates were prepared with this media and a 14 hr culture of *C. perfringens* NCYC 10239. Serial dilution of the culture were made in 0.1% peptone. Immediately prior to pouring the plates, the bottles were vigorously shaken for 20 sec. Pouring was carried out on a refrigerated surface to speed hardening of the medium. The plates were incubated anaerobically at 45°C for 24–36 hr. Percentage inhibition was calculated from the plate counts using the formula:

$$\% \text{ Inhibition} = \frac{\text{Control plate count} - \text{Treatment plate count}}{\text{Control plate count}} \times 100$$

RESULTS & DISCUSSION

Growth inhibition studies

In the inital study, all three strain of *C. perfringens* were completely inhibited by 150 ppm BHA in Fluid Thioglycollate Medium (FTM) when grown at 37°C. Growth at 50 and 100 ppm BHA was delayed or inhibited to a lesser degree (Table 1). The average plate count of the control cultures after 15 hr of incubation at 37°C was 2×10^8 cells/ml, whereas the cultures containing 200 and 400 ppm BHA had no viable organisms. Growth curves for the NCTC 10239 strain tested in media containing 0, 100 and 200 ppm BHA are given in Figure 1.

Subsequent experiments showed that 150 ppm was not always completely inhibitory. In several studies at 45°C, growth occurred in the solutions with 150 ppm BHA. The reasons for this variability are not known. The resistance (or susceptibility) of a microorganism is highly dependent on the age and health of the culture used (von Schelhorn, 1953). Other factors that cannot be totally accounted for

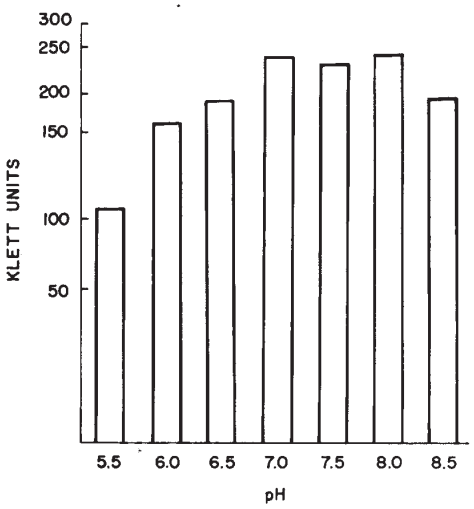

Fig. 2—The effect of pH on the inhibition of C. perfringens NCTC 10239 by 100 ppm BHA in Fluid Thioglycollate Medium at 45°C after 9 hours incubation.

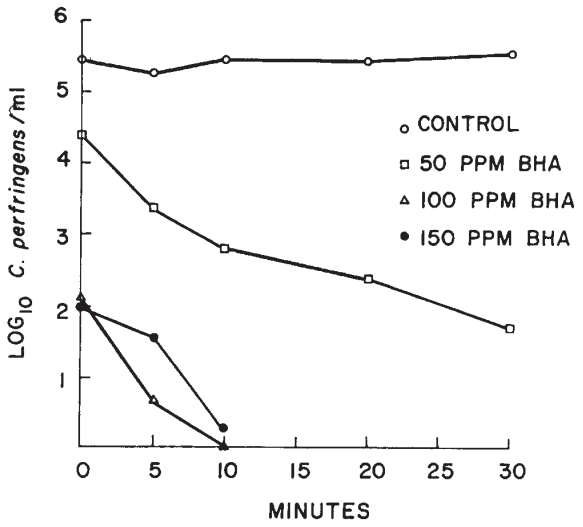

Fig. 3—Lethality rate of butylated hydroxyanisole on C. perfringens NCTC 10239 in a buffer system at 37°C as determined on Trypticase Sulfite Neomycin Agar.

may also have contributed to these variable results, such as differences in handling of samples and small differences in oxidation-reduction potential, pH, or temperature.

The ethanol used as a carrier for BHA was shown to have no deleterious effects on *C. perfringens* growth. Similarly, there was little difference in inhibition between cultures grown in media containing autoclaved versus filter-sterilized BHA. This indicates that BHA was not being volatilized and lost during the autoclaving process, nor changed in some way as to enhance or destroy its antimicrobial activity.

The activity and potential effectiveness of several food antimicrobials is dependent on the pH at which they are used. At all of the pH values tested the growth of *C. perfringens* was partially inhibited by 100 ppm BHA, but inhibition was greatest at the pH extremes where the organism was already under considerable stress (Figure 2). This increased inhibition does not necessarily indicate that BHA has optimal activity at these pH values, but rather, that there is an increase in the susceptibility of the organism to BHA at the pH extremes that were used.

Experiments using cultures of different ages indicated that the susceptibility of *C. perfringens* to BHA is age dependent. Fourteen to eighteen hour cultures grown in FTM at 45°C were more resistant to inhibition by BHA than cultures less than 14 or over 18 hr old. The 14 to 18 hr cultures also gave more consistent results for percentage inhibition by BHA. Plate counts of all the cultures were in the range of 2×10^8 to 3×10^8 cells/ml. Apparently the variability in inhibition by BHA that occurred with cultures of different ages was due to differences in metabolic rates (i.e., a fast metabolism in a young culture rendering it more susceptible to BHA inhibition) or to the presence of toxic metabolic end products in the medium. Further study on the actual mechanisms of BHA action on the individual cell may explain this finding.

Lethality rate of BHA

The lethality rate study was conducted to determine whether inhibitory concentrations of BHA were actually killing *C. perfringens* or simply preventing growth. The results of this study are shown in Figure 3. In the dilution buffer used for this study, the number of viable cells remained constant in the control throughout the test period, as indicated by plating on TSN agar. Upon addition of 50 ppm BHA to the medium, this number was immediately reduced by approximately one log cycle and by two and one-half log cycles after 30 min incubation. At 100 and 200 ppm BHA, the number of viable cells decreased even more dramatically, and by 10 min incubation, the plate counts showed that almost no viable cells were present. Although recovery of possibly injured cells may not have occured on the TSN agar, these results indicate that BHA was bactericidal rather than bacteriostatic.

BHA and other food antimicrobials

Foods are seldom, if ever, contaminated by only one type of microorganism. For this reason, more than one antimicrobial is often employed to insure complete inhibition of all the organisms present. This practice may also allow the use of smaller quantities of the individual antimicrobials. Occasionally, preservatives that are used together will enhance each other (von Schelhorn, 1953), but there is also the chance that one compound might reduce the other's activity. The tests using BHA with other antimicrobials were conducted for this reason. The results are shown in Table 2. It appears that none of the antimicrobials tested reduced the activity of BHA against *C. perfringens*. Rather, the data indicate that adding BHA may make it possible to reduce the concentrations of the other antimicrobials needed to achieve inhibition via a synergistic effect. This may be of interest to the food industry as a possible means of reducing the amounts of these antimicrobials used in food products. For instance, if testing of *Clostridium botulinum* with BHA and nitrite showed a response similar to that of *C. perfringens*, the amount of residual nitrite necessary for protection against botulism might be reduced, although BHA's usefulness in high fat products may be limited as discussed below. Likewise, the amount of sorbic acid or parabens might be reduced in other food products if BHA were added to these. Additional work using food systems is needed to determine whether the synergistic effects would exist in these processed foods as they do in a broth test system.

Effect of lipids on BHA activity

Unsaturated lipids are extremely reactive compounds

which undergo autoxidation by reaction with oxygen and subsequent formation of free radicals and hydroperoxides. Phenolic antioxidants such as BHA are active in preventing autoxidation by donating a hydrogen atom to the unstable lipid free radical, interfering with the chain reaction process that occurs (Dugan, 1976). The reaction between BHA and a lipid molecule in effect "uses up" the BHA molecule, making it no longer effective as an antioxidant. The same reaction could also result in destruction of the antimicrobial activity of BHA. Similarly, the hydrophobic nature of BHA and its solubility in lipophilic compounds might result in it being localized within the lipid portion of the medium, rendering it unavailable to act against those microorganisms which grow in the aqueous phase of the medium. An investigation of whether the interaction of BHA with lipid in a microbiological medium would affect the antimicrobial activity of BHA was, therefore, conducted.

The use of corn oil in TSN agar with Tween 80® as an emulsifier proved to be an effective method for production of an emulsion for this study. However, it was observed that Tween 80® caused considerable inhibition of *C. perfringens* growth. This inhibitory action of Tween 80® masked the effects of BHA inhibition at different lipid concentrations. It did appear that an increase in lipid concentration caused a reduction in the inhibition of *C perfringens* by BHA, indicating some interaction between BHA and the lipid. Antimicrobial activity still remained, however, as greater inhibition was observed for lipid samples containing BHA than for those without BHA.

Foods are extremely complex, varied systems, and several factors may control the effect of lipids on BHA inhibition. Therefore, it is not known if these observations can be applied directly to food products. The decreased antimicrobial activity of BHA due to Tween 80® and lipid indicates that a partitioning of BHA into the lipid phase of the medium is taking place. This same type of partitioning may also occur in a food product. If so, the potential for use of BHA as an antimicrobial is limited since it is currently allowed only in foods containing lipids, and these compounds appear to reduce its antimicrobial activity.

In conclusion, it has been shown that butylated hydroxyanisole does have a marked antimicrobial activity against *Clostridium perfringens* and will consistently inhibit its growth at a concentration of 200 ppm. This antimicrobial activity was bactericidal under the conditions of this study and also heat stable, withstanding the stress of autoclaving. Several factors appear to be important in the extent of inhibition of *C. perfringens* by BHA. These include the age and physiological state of the culture, temperature, the pH of the medium, and the presence of lipids. This last factor may limit the usefulness of BHA as an antimicrobial in lipid-containing food products.

Table 2—Inhibition of C. perfringens NCTC 10239 by several combinations of butylated hydroxyanisole, nitrite, sorbic acid and parabens[a]

	% Inhibition[b]			
Additive	0 ppm BHA	50 ppm BHA	100 ppm BHA	150 ppm BHA
Nitrite:				
0 ppm	0.0	5.6	16.1	79.1
50 ppm	0.0	0.0	39.9	100.0
100 ppm	7.4	6.5	71.7	100.0
Sorbic acid:				
0%	0.0	3.9	17.5	32.7
0.05%	17.8	20.4	37.9	76.3
0.075%	22.8	38.3	59.7	93.2
Parabens[c]:				
0%	0.0	—[d]	12.0	41.4
0.025%	8.6	18.2	38.8	93.6
0.05%	31.8	38.9	90.6	100.0

[a] Tested in Fluid Thioglycollate Medium at 45°C, inhibition determined by Klett colorimeter readings after 12 hr growth. Percentage inhibition was calculated by:

$$\% \text{ Inhibition} = \frac{(\text{Control} - \text{Control blank}) - (\text{Treatment} - \text{Treatment blank})}{(\text{Control} - \text{Control blank})} \times 100$$

[b] Each value was determined from the mean of three readings.
[c] A 3:1 mixture of the methyl and propyl esters of para-hydroxybenzoic acid
[d] Not determined

REFERENCES

Ayaz, M. 1975. Studies on the physiological characterization of staphylococci and the effects of butylated hydroxyanisole and butylated hydroxytoluene on growth and enterotoxin production of Staphylococcus aureus. Ph.D. thesis, Washington State University, Pullman, WA.

Chang, H.C. and Branen, A.L. 1975. Antimicrobial effects of butylated hydroxyanisole (BHA). J. Food Sci. 40: 349.

Chichester, D.F. and Tanner, F.W. Jr. 1968. Antimicrobial food additives. In "Handbook of Food Additives," Ed. Furia, T.E., p. 137. Chemical Rubber Co., Cleveland, OH.

Dework, F.M. Jr. 1972. Sporulation of Clostridium perfringens Type A in vacuum-sealed meats. Appl. Microbiol. 24: 834.

Dugan, L. Jr. 1976. Lipids. In "Principles of Food Science, Part 1; Food Chemistry," Ed. Fennena, O.R., p. 139. Marcel Dekker, Inc., NY.

Forrest, J.C., Aberle, E.D., Hedrick, H.B., Judge, M.D. and Merkel, R.A. 1975. "Principles of Meat Science." W.H. Freeman and Co., San Francisco.

Griffin, W.C. and Lynch, M.J. 1968. Surface active agents. In "Handbook of Food Additives," Ed. Furia, T.E., p. 413. Chemical Rubber Co., Cleveland, OH.

Robach, M.C., Smoot, L.A. and Pierson, M.D. 1977. Inhibition of Vibrio parahaemolyticus 04:K11 by butylated hydroxyanisole, J. Food Prot. 40: 549.

Shih, A.L. and Harris, N.D. 1977. Antimicrobial activity of selected antioxidants. J. Food Prot. 40: 520.

von Schelhorn, M. 1953. Efficacy and specificity of chemical food preservatives. Food Technol. 7: 97.

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