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# Vapor Analysis of Fermented Spanish-type Green Olives by Gas Chromatography

SUMMARY—*Five major components were detected gas chromatographically in the head-space vapor (HSV) of Spanish-type green olives fermented by pure cultures of* Lactobacillus plantarum, Pediococcus cerevisiae *and* Leuconostoc mesenteroides. *Three of these compounds were identified as acetaldehyde, methyl sulfide, and ethanol. The same compounds were present in unfermented olives but in different amounts. Olives that had undergone a natural fermentation contained the above five compounds, and, in addition, a varying number of other compounds. These results indicated that HSV analysis may be a rapid method for detecting volatile end products resulting from the metabolism of various microorganisms. A high ethanol content was found in olive brines that contained a predominance of yeasts. Abnormal fermentations gave unique HSV profiles, one of which indicated a high level of 2-butanol. Methyl sulfide was found to be a major odor component of fermented as well as unfermented olives. Acetaldehyde and ethanol contributed secondarily to the odor. Primary contributions of fermentation by the above lactic acid bacteria to the flavor of olives were: (1) production of a desirable level of acidity, and (2) utilization of fermentable sugars to the exclusion of microorganisms which produce metabolic end products with undesirable flavor characteristics.*

## INTRODUCTION

THE PRESERVATION of fruits and vegetables by brining has been practiced for centuries, with comparatively few technological advances. Organoleptic qualities of products preserved in this manner, such as cucumbers, olives and cabbage, are greatly influenced by the variable nature of microbial activity. Recent efforts have been made to control more closely the quality of such products. This is done by regulating the microbial flora with the addition of pure cultures of lactic acid bacteria to heat-shocked cucumbers and olives (Etchells et al., 1964, 1966).

The concentration of lactic acid, measured as titratable acidity, indicates the extent to which lactic acid bacteria were active in the fermentation of olives. It is an important criterion used to determine the success of olive fermentations. Other end products of microbial metabolism, whether present in large or trace amounts, may be of great significance with respect to organoleptic properties of olives, especially flavor.

Alcohols, esters, aldehydes, ketones, etc., as well as acids, are among end products known to be formed by microorganisms which are competitive with lactic acid bacteria in brined olives. Clostridia, coliforms, yeasts, and molds have been associated with spoilage problems of fermented olives (Vaughn, 1954; Vaughn et al., 1943) and may produce many of the previously mentioned end products.

Gas chromatographic analysis of head-space vapors has been successfully employed in determining volatile constituents of many foods including vegetables (Buttery et al., 1961), milk (Bassette et al., 1963), and alcoholic beverages (Kepner et al., 1964). This work was undertaken to establish the value of the head-space vapor method for detecting volatile compounds as an indication of the types of microorganisms active in the fermentation of olives. It was further desired to ascribe relative significance of the components detectable by the method to the odor of fermented olives.

## EXPERIMENTAL

### Fermented olives

The pure-culture fermented Spanish-type green olives (Manzanillo variety) examined in this study were samples from a previous study (Etchells et al., 1966). The processing of these samples involved lye treatment to remove bitterness, removal of the lye by leaching in water, heat-shocking the olives at 74°C for 3 min in a water bath, followed by packing into 0.5-gal glass jars with a pasteurized and cooled 40° salometer brine (10.6% NaCl). The addition of 2.5 ml of 85% lactic acid to each jar provided an initial pH of 7.0–7.4.

The jars scheduled for inoculation received 8 ml of a 30 hr culture of the designated species of lactic acid bacterium which had been grown at 32°C in Trypticase Sugar Broth (BBL). After incubating the samples at 21–24°C for 7.5 months, the olives were evaluated as to flavor and other organoleptic

and physical qualities as described by Etchells et al. (1966). Assays of the brines for titratable acidity, pH, and transmittance, and microscopic examination of brines were determined according to Etchells et al. (1964).

A portion of the olives was repacked into quart jars, with the addition of one ml of a one percent aqueous Merthiolate solution to preclude further microbial activity. These jars were then held at 5°C until being assayed by gas chromatography.

### Head-space vapor analysis

Olives were removed from refrigeration and immediately pitted with a hand-operated mechanical olive pitter. Ten grams of finely diced olive tissue, 20 ml brine, 40 ml distilled and cooled (5°C) water, 30 g sodium sulfate and a 1 in. Teflon-coated magnetic bar were added to a 125 ml erlenmeyer flask. Two ml of 0.01% (v/v) 2-butanol in water were added to serve as an internal standard.

A rubber septum was fitted onto the flask and, while the contents of the flask were still cool, 30 ml of vapor were removed through the septum with a 50-ml syringe to provide sufficient vacuum to prevent excessive pressure inside the flask upon heating. The flask was submerged up to the neck in a water bath held at 60°C on a hot plate magnetic stirrer.

After holding the flask in the bath for 15 min, with the magnetic bar slowly stirring, 5 ml of vapor were removed with a 6-ml disposable syringe (Monoject 506 S). The syringe, fitted with a 25-gauge needle, was filled and emptied back into the flask four times. The fifth filling was injected into a Barber-Colman Model 10 gas chromatograph equipped with a hydrogen flame detector. A stream splitter allowed 50% of the injected sample to enter the detector and 50% to emerge through the collector port for odor monitoring. The column consisted of a 9-ft U-shaped glass tubing, 1/4-in. i.d., packed with 5% Carbowax 20 M on 60/80 mesh GC-22 Firebrick. The operating conditions were: column, 75°C; detector, 190°C; flash heater, 170°C; nitrogen carrier gas, 23 ml/min.

Relative peak heights of chromatograms were expressed as the peak heights for unknown components divided by the peak height for 2-butanol which was added to each sample. Adjustment in attenuation was necessary to record all of the peaks for a sample and calculations included multiplication by the appropriate factor. The attenuation was the same, however, for respective peaks obtained from different samples.

Assays were performed in duplicate. Vapor components arising from the sampling syringe, flask, reagents, etc. involved in the assay were negligible and did not significantly influence relative peak heights of the major components reported herein.

Identification of head-space vapor compounds was made by comparing their retention times with known compounds on three columns. These columns and the conditions were: (1) Carbowax 20 M, as described above; (2) Diisodecyl phthalate, 5%, on 60/80 mesh Firebrick; 9-ft U-shaped, 1/4 in. i.d. glass column; 75°C; $N_2$ carrier gas, 23 ml/min; (3) Polyethylene glycol 600, 15%, on 60/80 mesh Firebrick; 6-ft U-shaped, 1/4-in. i.d. glass column; 75°C; $N_2$ carrier gas, 23 ml/min.

The syringe reaction technique of Hoff et al. (1964) was employed to establish which peaks represented aldehydes or ketones. This procedure involved coating the inside of 5-ml ground-glass hypodermic syringes used to sample the head-space vapor with a solution of either potassium permanganate or hydroxylamine. The absence of a peak after this treatment indicated that the compound was either an aldehyde or a ketone.

### Vacuum distillation

Fermented olives were pitted and 200 g of tissue along with 100 ml of cover brine from the olives and 200 ml of distilled water were placed in a 5-L flask. This flask was connected to a high vacuum system containing three traps in series. These traps, in order, were: (1) ice-salt, (2) dry ice-acetone and (3) liquid nitrogen. The assembly was similar to that described by Aurand et al. (1965); but the sample was not swept with nitrogen.

The sample was distilled under a pressure of 35$\mu$ of mercury for 3 hr. with the distillation flask being maintained at about 25°C. Approximately 40% of the sample volume was distilled by this procedure.

### Mass spectrometry

Mass spectrometry was performed with a coupled Barber-Colman Model 5000 gas chromatograph and a Bendix Model *12*-107 Time-of-Flight Mass Spectrometer. The system was similar to that described by Teranishi et al. (1963). The GLC column was a 6-ft, 1/4-in. stainless steel tubing packed with 5% Carbowax 20 M on 60/80 mesh Chromosorb G. Components eluted from the GLC column were observed as the mass spectral output on an oscilloscope and the mass spectral pattern recorded by rapid scan at appropriate times.

Prior to the run, contents of the liquid nitrogen trap from vacuum distillation were trapped in a U-shaped, 6-in. long, 1/4-in. diameter, stainless steel pre-column of 5% Carbowax 20 M on 60/80 mesh Chromosorb G. This transfer was effected by holding the pre-column in liquid nitrogen while sweeping in the contents of the trap from vacuum distillation with nitrogen gas. The pre-column was then removed from liquid nitrogen and coupled with the injection port of the gas chromatograph. The system was swept with helium as the carrier gas. The temperature of the GLC column was 50°C initially and was programmed at 3°C per min up to a final temperature of 150°C.

## RESULTS & DISCUSSION

### Pure-culture fermented olives

Gas chromatography of the head-space vapor of pure-culture fermented olives on Carbowax 20 M resulted in chromatogram profiles possessing 5 major peaks. A typical chromatogram obtained from a fermentation by *L. plantarum* is shown in Figure 1A. Peak No. 6 represents 2-butanol which was added as an internal standard. Profiles from olives pure-culture fermented by *P. cerevisiae* and *L. mesenteroides*, as well as those from unfermented (Fig. 1B) and natural fermented (Fig. 1C) olives, indicated the presence of these same 5 components on the basis of retention times.

Compounds represented as peak No. 1, 2 and 5 (Fig. 1) were identified as acetaldehyde, methyl sulfide and ethanol, respectively, by comparison of retention times with known compounds on three different columns (Experimental). The

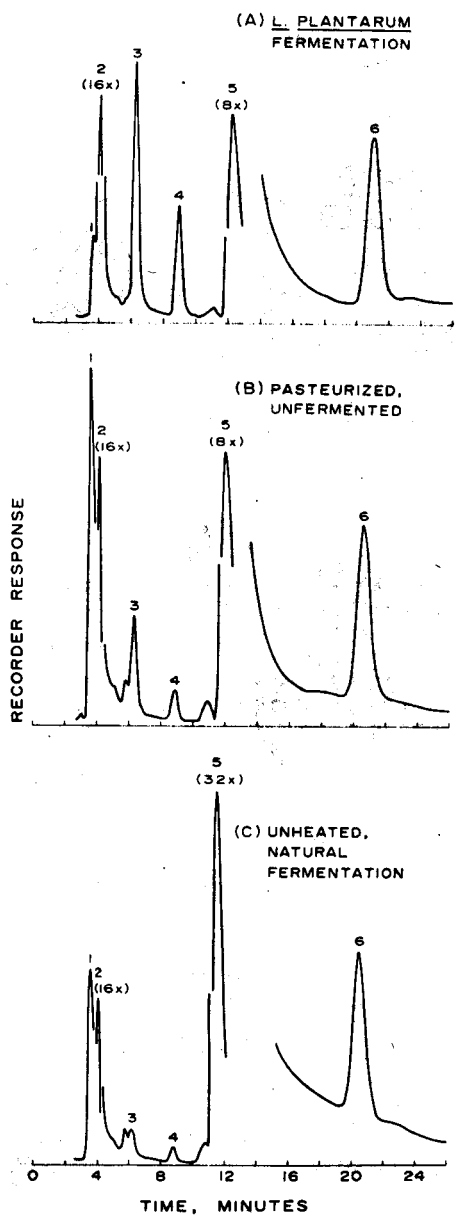


*Fig. 1—Head-space vapor GLC profiles of (A) pure-culture fermented; (B) pasteurized, unfermented; and, (C) naturally fermented olives. Numbers above peaks were identified as (1) acetaldehyde, (2) methyl sulfide, (3) unknown, (4) unknown, (5) ethanol, and (6) 2-butanol added as an internal standard. The attenuation was 2 X except as noted in parentheses above peaks. The column was Carbowax 20 M and was operated at 75°C.*

Table 1—Vapor and brine analyses of fermented olives.

| Fermentation Type | Vapor analysis[1] | | | | | Brine analysis | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Acetaldehyde | Methyl sulfide | Peak No. 3 unknown | Peak No. 4 unknown | Ethanol | pH | Acidity as lactic | Transmittance | Microscopic examination |
| | | | | | | | % | % | |
| *Lactobacillus plantarum* | 0.48 | 9.82 | 1.43 | 0.64 | 4.60 | 3.8 | 1.10 | 7 | Abundant short rods only |
| *Pediococcus cerevisiae* | 0.63 | 8.41 | 0.58 | 0.27 | 4.85 | 4.6 | 0.47 | 25 | Abundant pediococci only |
| *Lactobacillus plantarum* + *Pediococcus cerevisiae* | 0.58 | 11.30 | 1.61 | 0.78 | 6.05 | 3.8 | 1.08 | 7 | Abundant short rods, few pediococci |
| *Leuconostoc mesenteroides* | 0.72 | 9.43 | 0.63 | 0.70 | 18.87 | 5.1 | 0.34 | 35 | Leuconostoc, in chains, not abundant |
| *Leuconostoc mesenteroides*[2] | 1.64 | 10.77 | 0.19 | 0.04 | 7.89 | 6.7 | 0.08 | 75 | Few yeasts and rods, not established |
| Pasteurized, uninoculated control | 1.99 | 11.28 | 0.56 | 0.17 | 6.53 | 6.2 | 0.13 | 77 | No microbes seen |
| Unheated, natural fermentation | 1.16 | 8.44 | 0.16 | 0.09 | 36.31 | 5.8 | 0.13 | 67 | Yeasts only |
| Av. % difference from mean of duplicate assays for 24 samples | 4.6 | 8.4 | 2.9 | 3.2 | 6.0 | | | | |

[1] Relative peak heights from head-space vapor analysis; average of duplicate assays.
[2] This sample of olives differed from the others in that it did not receive 2.5 ml of 85% lactic acid per 0.5-gal jar as described in the Experimental section.

identification of peak No. 1 as acetaldehyde was further verified by the syringe reaction methods of Hoff et al. (1964) for the removal of aldehydes.

The presence of methyl sulfide was confirmed by vacuum distillation, trapping with liquid nitrogen, and subsequent mass spectrometry. Concentrations of the other volatiles from this distillation were insufficient for identification by mass spectrometry.

While HSV profiles from the various olive samples indicated qualitative similarity, quantitative differences in the 5 major vapor components among fermentations existed, as evidenced by peak heights (Fig. 1). Relative peak heights of HSV profiles were determined to serve as an index of these quantitative differences (Table 1). The reproducibility of relative peak heights, as determined by duplicate assays, is indicated in Table 1, and was considered sufficient for detecting major quantitative variations in vapor composition of olive samples.

Brine analyses which are performed routinely to indicate the success of olive fermentations (Vaughn et al., 1943; 1954) are reported in Table 1 for comparison with the HSV data.

Olive samples inoculated with *L. Plantarum* and *P. cerevisiae* resulted in successful fermentations as determined by the abundant growth of these microorganisms with resulting pH and acidity values typical of a desirable product (Table 1). *L. mesenteroides* was less dependable as the fermenting bacterium as was reported by Etchells et al. (1966).

One sample which fermented to some extent and another which failed to ferment are reported in Table 1. The sample which failed to ferment did not receive 2.5 ml of lactic acid prior to inoculation as did the other samples reported in Table 1. Thus, fermentation failure was probably related to high initial pH. No apparent fermentation occurred in the pasteurized (i.e., heat-shocked at 74°C for 3 min prior to packing) olives. The unheated, natural fermentation resulted in a predominance of yeasts with the development of little acidity.

Several correlations existed between relative peak heights and microbial activity among the olive samples reported in Table 1. Acetaldehyde content apparently was reduced as a consequence of fermentation by lactic acid bacteria, based on comparison with the pasteurized, unfermented sample. Metabolism of this compound in milk by certain cultures of *Leuconostoc citrovorum* has been reported (Lindsay et al., 1965) and may explain its reduction in the present instance.

Ethanol was present in all products; but its increased concentration in brines with high populations of yeasts was apparent. Fermentation by *L. mesenteroides* also resulted in a high level of ethanol, which is consistent with the end products expected for this heterofermentative bacterium. Fermentations by the homofermentative bacteria *L. plantarum* and *P. cerevisiae* resulted in comparatively low levels of ethanol.

Methyl sulfide content in the vapor did not vary greatly among the various fermentation types. Similar levels were present in the vapor of the unfermented and the fermented olives.

Relative peak heights for the unidentified vapor components, indicated as peak No. 3 and 4, also served to characterize the fermentation type (Fig. 1, Table 1). Peak heights for component No. 3 were characteristically high when *L. plantarum* was the fermenting bacterium. The most striking observation concerning component No. 4 was its relatively low concentration in the vapor of unfermented olives and those that underwent natural fermentation with a predominance of yeast activity.

### Commercially brined olives

Samples of commercially brined olives that had undergone a natural fermentation gave HSV profiles that indicated variations in qualitative as well as quantitative composition of volatile compounds (Fig. 2). Most commercial brines gave a HSV profile similar to that shown in Figure 2A. The same five major peaks were present in the chromatograms of these brines as were found with the pure culture fermented olives. In addition, several other peaks were evident.

The ethanol peak varied greatly among the commercial brines, presumably reflecting varying degrees of activity by yeasts and heterofermentative lactic acid bacteria. The very low level of acetaldehyde in the commercial brines may have been due to dissipation of this highly volatile compound during handling of the olives by the packer.

Two samples of commercially brined olives considered to be atypical are represented by the HSV profiles in Figure 2B and 2C. In several samples there was a very high level of 2-butanol, ironically the compound used earlier as an internal standard (Fig. 2B). Identity of 2-butanol was made on the basis of retention time on the three GLC columns as described in the Experimental section.

The apparently high concentration of

2-butanol in the brine strongly indicates that appreciable microbial activity in addition to or to the exclusion of lactic acid bacteria occurred in this lot of olives. The odor of these olives, although acceptable, was considered "unclean" in comparison with the pure culture fermented olives.

Another sample of commercial olives had a repulsively strong butyric acid odor and was designated as "malodorous" on the basis of the description by Vaughn (1954). These olives also were soft, and definitely unacceptable for consumer use. The characteristic volatiles of fermented olives were greatly reduced or absent from this sample (Fig. 2C). It seems likely that these olives had been washed several times in an effort to remove the unpleasant odor, but without success as the odor of butyric acid is detectable at very low levels.

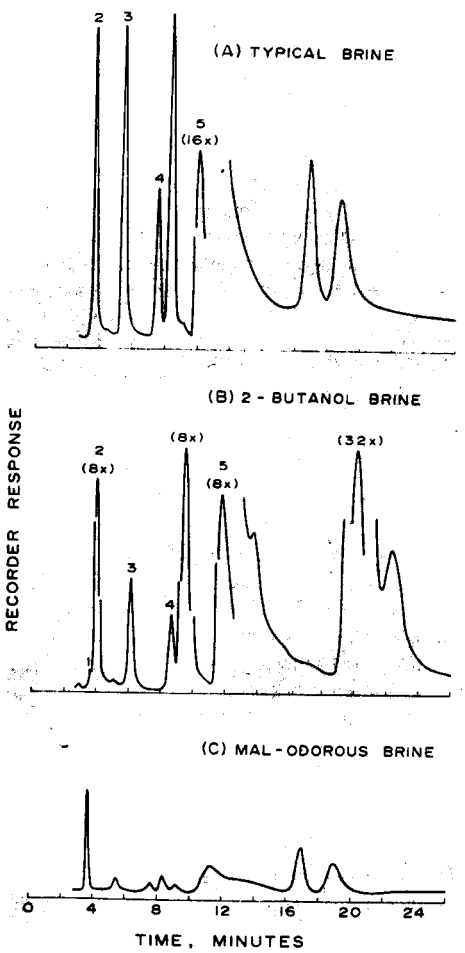


*Fig. 2—Head-space vapor GLC profiles of naturally fermented olives from commercial sources showing variations that existed in volatile compounds. Sample (A) was a typical brine with a pleasant odor; (B) a brine that contained a high level of 2-butanol (peak attenuated 32 X); and, (C) a brine that possessed a strong odor of butyric acid. The attenuation was 2 X except as noted. The column was Carbowax 20 M and was operated at 75°C.*

Table 2—Fractionation of fermented olive volatiles by vacuum distillation.

| Trap | Odor description |
|---|---|
| Ice-salt | Weakly alcoholic |
| Dry ice-acetone | Strongly alcoholic |
| Liquid nitrogen | Strong fermented olive, methyl sulfide-like |
| Residue | Weak fermented olive, nearly bland |

### Major odor components

The volatile compounds of olives fermented by *L. plantarum* were fractionated into two groups by vacuum distillation (Table 2). The ice-salt and dry ice-acetone traps contained alcoholic odors. The liquid nitrogen trap contained what was considered the primary odor of fermented olives.

Odor-monitoring of GLC effluents from chromatographing samples from the liquid nitrogen trap revealed only two components that could be detected by smelling. These components were methyl sulfide and acetaldehyde as indicated by retention times.

Methyl sulfide odor could be detected when chromatographing on any of the three stationary phases used in this study. The odor of acetaldehyde could be detected only when chromatographing on diisodecylphthalate, as the other two columns did not adequately resolve this component from methyl sulfide. When the two components emerged in mixtures, only the odor of methyl sulfide was detected.

Odor-monitoring of GLC effluents from head-space vapor samples revealed only one component in effluents, methyl sulfide, that could be detected by smelling. This compound was concluded to be a major odor component of brined olives as the compound alone in certain concentrations gave an odor very similar to that of the olives. Thus, brined olives are among the increasing number of foods which have been reported to contain flavor threshold levels of methyl sulfide. Other such foods include tomatoes (Miers, 1966), potatoes (Gumbmann et al., 1964) and Swiss cheese (Langler et al., 1967).

Similar concentrations of methyl sulfide in the head-space vapor of brined olives, whether fermented or unfermented, indicates that it is derived from the olive under normal brining conditions.

Acetaldehyde, and to a lesser extent ethanol, were considered to contribute secondarily to the odor of brined olives. Unidentified components indicated as peak no. 3 and 4, and other compounds may also modify the basic odor ascribed to methyl sulfide.

Samples of olives were subjected to informal taste panel evaluation. Pure-culture fermented olives were described as having a "clean" fermented olive flavor. The term "clean" was defined as the absence of off-flavors. The flavor of the unfermented product was rated as being more bland and lacking sharpness, probably due to the lack of acidity. The odors of the fermented and unfermented products were similar.

From the above observations it was concluded that the primary contributions of fermentation by lactic acid bacteria to the flavor of olives include: (1) the production of a desirable level of acidity; and (2) utilization of fermentable sugars to the exclusion of microorganisms which produce metabolic end products with undesirable flavor characteristics.

## REFERENCES

Aurand, L.W., Singleton, J.A., Bell, T.A. and Etchells, J.L. 1965. Identification of volatile constituents from pure-culture fermentation of brined cucumbers. *J. Food Sci.* **30,** 288–295.

Bassette, R., Ozeris, S. and Whitnah, C.H. 1963. Direct chromatographic analysis of milk. *J. Food Sci.* **28,** 84–90.

Buttery, R.G. and Teranishi, R. 1961. Gas-liquid chromatography of aroma of vegetables and fruit. Direct injection of aqueous vapors. *Anal. Chem.* **33,** 1439–1441.

Etchells, J.L., Costilow, R.N., Anderson, T.E. and Bell, T.A. 1964. Pure culture fermentation of brined cucumbers. *Appl. Microbiol.* **12,** 523–535.

Etchells, J.L., Borg, A.F., Kittel, I.D., Bell, T.A. and Fleming, H.P. 1966. Pure culture fermentation of green olives *Appl. Microbiol.* **14,** 1027–1041.

Gumbmann, M.R. and Burr, H.K. 1964. Volatile sulfur compounds in potatoes. *J. Agr. Food Chem.* **12,** 404–408.

Hoff, J.E. and Feit, E.D. 1964. New technique for functional group analysis in gas chromatography. Syringe reactions. *Anal. Chem.* **36,** 1002–1008.

Kepner, R.E., Maarse, H. and Strating, J. 1964. Gas chromatographic head space techniques for the quantitative determination of volatile components in multicomponent aqueous solutions. *Anal. Chem.* **36,** 77–82.

Langler, J.E., Libbey, L.M. and Day, E.A. 1967. Identification and evaluation of selected compounds in Swiss cheese flavor. *J. Agr. Food Chem.* **15,** 386–391.

Lindsay, R.C., Day, E.A. and Sandine, W.E. 1965. Green flavor defect in lactic starter cultures. *J. Dairy Sci.* **48,** 863–869.

Miers, J.C. 1966. Formation of volatile sulfur compounds in processed tomato products. *J. Agr. Food Chem.* **14,** 419–423.

Teranishi, R., Corse, J.W. McFadden, W.H., Black, D.R. and Morgan, A.T. Jr. 1963. Volatiles from strawberries. I. Mass spectral identification of the more volatile components. *J. Food Sci.* **28,** 478–483.

Vaughn, R.H., Douglas, H.C. and Gililland, J.R. 1943. Production of Spanish-type green olives. Calif. Agr. Expt. Sta. Bull. 678.

Vaughn, R.H. 1954. Lactic acid fermentation of cucumbers, sauerkraut and olives. In "Industrial Fermentations," eds. Underkofler, L.A. and Hickey, R.J. Vol. 2. Chemical Publishing Co., Inc., New York.

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