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TECHNICAL REPORT
NATICK/TR-84/035

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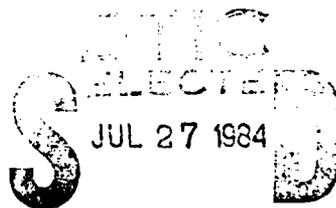
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ANTIOXIDANTS IN SPICES: LITERATURE SURVEY AND IDENTIFICATION IN CLOVE

BY
ROSLYN E. KRAMER

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REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM	
1. REPORT NUMBER NATICK/TR-84/035	2. GOVT ACCESSION NO. A142 377	3. AUTHOR'S CATALOG NUMBER	
4. TITLE (and Subtitle) ANTIOXIDANTS IN SPICES: LITERATURE SURVEY AND IDENTIFICATION IN CLOVE		5. TYPE OF REPORT & PERIOD COVERED Technical Report-Final	
7. AUTHOR(s) Roslyn E. Kramer		6. PERFORMING ORG. REPORT NUMBER	
9. PERFORMING ORGANIZATION NAME AND ADDRESS Science & Advanced Technology Laboratory/STRNC-YSA US Army Natick Research & Development Center Natick, Massachusetts 01760		8. CONTRACT OR GRANT NUMBER(s)	
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Natick Research & Development Center ATTN: STRNC-Z Natick, Massachusetts 01760		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 1L162724AH99BA023	
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office) Same as above.		12. REPORT DATE June 1984	
		13. NUMBER OF PAGES 24	
		15. SECURITY CLASS. (of this report) Unclassified	
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE	
16. DISTRIBUTION STATEMENT (of this Report) Approved for public release; distribution unlimited.			
17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report) N/A			
18. SUPPLEMENTARY NOTES None			
19. KEY WORDS (Continue on reverse side if necessary and identify by block number) CHROMATOGRAPHY ANTIOXIDANTS ULTRAVIOLET CLOVE INFRARED SPICES MASS SPECTROMETRY SHELF LIFE LIQUID CHROMATOGRAPHY STORAGE			
20. ABSTRACT (Continue on reverse side if necessary and identify by block number) Through the use of thin-layer chromatography, ultraviolet (UV), infrared (IR), mass spectrometry (MS), and liquid chromatography (HPLC), gallic acid and eugenol were identified as the two major antioxidants in clove. The amounts of gallic acid and eugenol were determined to be 1.26 g and 3.03 g, respectively, in 100 g of clove.			

PREFACE

This study was undertaken to identify any antioxidants that could be isolated from spices.

The author would like to thank Dr. Ahmed Mabrouk for his help whenever needed; Dr. Edmon Wong, Department of Scientific and Industrial Research, Palmerston, New Zealand; Dr. Aaron Bluhm for the IR spectrometry; Mr. Maurice Bazinet and Dr. Donald Robertson for the mass spectrometry; Solomon Bishov for the testing of the first fractions for antioxidant activity; and Mr. Chris Filippi and his colleagues at Waters Associates for their invaluable help in HPLC. This work was carried out under Project No. 1L162724AH99BA023 entitled, "Antioxidant Systems for Improved Stability".

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IDENTIFICATION OF ANTIOXIDANTS IN SPICES: LITERATURE SURVEY AND IDENTIFICATION IN CLOVE

INTRODUCTION

There is a recognized need for military rations to have the greatest shelf life attainable. Contingency reserves should be able to withstand the stresses of long term storage that may be imposed upon them. These food reserves may be in remote locations about the world and their rotation at too-frequent intervals due to inadequate shelf life represents an undesirable expense. Commercial counterparts to such rations are not produced for maximum shelf life because the items are geared to production cycles, limited inventories, and protection from temperature extremes.

One of the principal pathways by which shelf life is shortened is by oxidation of lipids and subsequent reaction of the compounds produced. Antioxidants are present in unprocessed foods but once processing begins the antioxidants' effectiveness may be insufficient to assure product acceptability, or the processing itself may destroy them. Food technologists have usually added antioxidants to counter these effects and chemists have extended their knowledge of mechanisms by which different antioxidants function in foods. In recent years there has been rising concern over the effect of some purely synthetic

antioxidants on the ultimate health of the consumer. Although such compounds are widely used in the food industry, we need effective alternatives to compounds such as Butylated Hydroxyanisole (BHA), Butylated Hydroxytoluene (BHT), and more recently Tertiary-Butyl Hydroquinone (TBHQ). Since a number of traditional recipe ingredients have recognized antioxidant activity, we need to extend our knowledge of the active principals.

Despite the knowledge that spices help preserve food, little work has been done on identifying their antioxidants. Chipault (1,2,3,4) in the 1950's investigated the antioxidative properties of spices. In the 1960's Herrmann (5,6) isolated an antioxidant in rosemary and sage that he called labiatic acid and Brieskorn,

¹ J.R. Chipault, G.R. Mizuno, J.M. Hawkins, and W.O. Lundberg, "The Antioxidant Properties of Natural Spices", Food Research, 17:46 (1952).

² J.R. Chipault, G.R. Mizuno, and W.O. Lundberg, "Antioxidant Properties of Spices in Oil-in-Water Emulsions", Food Research, 20:443 (1955).

³ J.R. Chipault, G.R. Mizuno, and W.O. Lundberg, "The Antioxidant Properties of Spices in Foods", Food Technology, 10:209 (1956).

⁴ J.R. Chipault, "32 Spices Gaged as Antioxidants", Food Engineering, April 1957, 134.

⁵ K. Herrmann, "The Antioxidative Effect of Labiate Drugs and Labiate Acid (Labiatic Tannic Acid) Contained Within Them", Z. Lebensm.-Unters.-Forsch, 116:224 (1962).

⁶ K. Herrmann, "Phenolic Compounds of Plants as Natural Antioxidants", Fette Seifen Anstrichmittel, 75:499 (1973).

co-workers, and Doemling (7,8,9) identified carnosol and carnosic acid in the same two herbs. There has been increased research in the 1970's, especially in Japan where Hirahara, Takai, and Iwao (10) found a variation in the antioxidant effects of spices depending on the source and type of the spice, time of harvest, and treatment. These effects varied with slight differences in experimental conditions, including the type of food, water content, emulsified states, preservation conditions and method of determining deterioration rates. The investigators went on to study the effect of certain spices and spice extracts on soybean, olive, sesame, and linseed oils. Watanabe and Ayano (11) prepared and tested water- and ethanol-soluble fractions as well as

⁷C.H. Brieskorn, A. Fuchs, J.B. Bredenberg, J.D. McChesney, and E. Wenkert, "The Structure of Carnosol", *Journal of Organic Chemistry*, 29:2293 (1964).

⁸C.H. Brieskorn, H.J. Doemling, "Carnosic Acid: An Important Antioxidant in Rosemary and Sage Leaves", *Zeitschrift fuer Lebensmittel-Untersuchung und-Forschung*, 141:10 (1969).

⁹C.H. Brieskorn, H.J. Doemling, "Natural and Synthetic Derivatives of Carnosic Acid", *Archiv. der Pharmazie*, 302:641 (1969).

¹⁰Hirahara, F., Takai, Y., and Iwao, H., "Antioxidative Activity of Various Spices on Oils and Fats", *The Japanese Journal of Nutrition*, 32:1 (1974).

¹¹Watanabe, Y. and Ayano, Y., "The Antioxidative Activities of Distilled Water Soluble and Ethanol Soluble Fractions from Ground Spices", *The Japanese Journal of Nutrition*, 27:181 (1974).

the ground state of 10 spices. Yutaka Salto (12) has worked and written extensively on spices, including a review on the progress of research on their antioxidant properties through 1977. He concluded that rosemary, sage, thyme, marjoram, and oregano among the herbs and clove, ginger, nutmeg, and mace among the spices have strong antioxidant effects. Identification of the substances contained in these spices that cause the effects is limited to those substances found in rosemary and sage most recently by Chang, Ostric-Matijasevic, Hsieh, and Huang (13, 14) and Wu, Lee, Ho, and Chang (15). Bishov, Henick, and co-workers

¹²Salto, Yutaka, "Antioxidant Properties of Spices, *Abura Kagaku*, 26:754 (1977).

¹³Chang, S.S., Ostric-Matijasevic, B., Hsieh, O.A.L., and Huang, S.-L., "Natural Antioxidants from Rosemary and Sage", *Journal of Food Science*, 42:1102 (1977).

¹⁴Chang, S.S., Ostric-Matijasevic, B., Huang, C.-L., and Hsieh, O.A.L., "Method of Producing an Antioxidant Composition from Rosemary and Sage", U.S. Patent No. 3905266 (1976).

¹⁵Wu, J.W., Lee, M.-H., Ho, C.-T., and Chang, S.S., "Elucidation of the Chemical Structures of Natural Antioxidants Isolated from Rosemary", *Journal of the American Oil Chemists' Society*, 59:339 (1982).

(16-21) of these laboratories investigated the oxidation of fat in model systems, the antioxidant effect of the components of freeze-dried foods and the synergism of various antioxidants when used together. Bishov, Masuoka, and Kapsalis (22) found high antioxidant activity in clove and so clove was picked for the present study, which was initiated to identify the compounds responsible for its antioxidant activity.

- ¹⁶Bishov, S.J., Henick, A.S., and Koch, R.B., "Oxidation of Fat In Model Systems Related to Dehydrated Food", *Food Research*, **25**:174 (1960).
- ¹⁷Bishov, S.J., Henick, A.S., and Koch, R.B., "Oxidation of Fat In Model Systems Related to Dehydrated Foods II Composition and Position of Dispersed Lipid Components and Their Effect on Oxidation Rates", *Journal of Food Science*, **26**:178 (1961).
- ¹⁸Bishov, S.J. and Henick, A.S., "A Gas Chromatographic Method for Continuous Accelerated Study of O₂ Uptake in Fats", *Journal of the American Oil Chemists' Society*, **43**:477 (1966).
- ¹⁹Bishov, S.J., Masouka, Y., and Henickm A.S., "Fat Quality and Stability In Dehydrated Proteinaceous Food Mixes", *Food Technology*, **21**:148A (1967).
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- ²²Bishov, S.J., Masuoka, Y., and Kapsalis, J.G., "Antioxidant Effect of Spices, Herbs, and Protein Hydrolyzates In Freeze-Dried Model Systems: Synergistic Action with Synthetic Phenolic Antioxidants", *Journal of Food Processing and Preservation*, **1**:153 (1977).

INSTRUMENTATION AND MATERIALS

The ultraviolet analysis was performed on a Cary Model 15 Recording Spectrophotometer. The Infrared analysis was performed on a Nicolet FTIR Model 7000 Series Spectrometer. The mass spectrometers used were: CEC Model 110 High Resolution Mass Spectrometer, and the SCIEX, Ltd. Model TAGA-6000 MS/MS Atmospheric Pressure Ionization Source. The liquid chromatograph was a Waters High Performance Liquid Chromatograph equipped with Model 6000A solvent delivery system, Model 660 solvent programmer, Model U6K universal injector and Model 450 UV detector.

The materials were obtained from the following: ground clove (McCormick Industrial Flavor Division); petroleum ether, ethyl acetate, ethyl ether, α, α' -dipyridyl (certified), purified ferric chloride anhydrous, linoleic acid, 2-propanol, and acetic acid (Fisher Scientific Co.); ethanol, chloroform, and methanol (Burdick & Jackson); chloroform and eugenol (Eastman); propyl gallate (NIPA Laboratories); gallic acid (Pfalz & Bauer); and polygram polyamide - 6 UV254 for thin-layer chromatography, pre-coated plastic sheets 20 x 20 cm (Brinkman Instruments, Inc.).

EXPERIMENTATION

Extraction Procedure

150 g ground clove were packed into a glass chromatography column (500 mm x 35 mm). Two liters of petroleum ether were percolated through the column from a reservoir above to remove any fat and much of the color pigments. This was followed by two liters of 80% ethanol to extract the phenolic compounds and sugars. The ethanol extract was concentrated on a rotary evaporator and extracted three times with ethyl acetate to remove the phenolic compounds and polar organics. Finally, the remaining ethanol solution was extracted three times with ethyl ether to remove the non polar organic compounds. All reagents

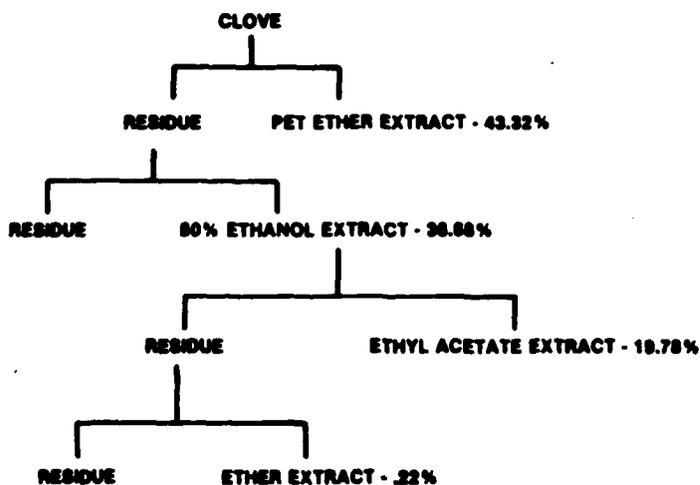


Figure 1. Scheme for the extraction of antioxidants from clove.

were tested for peroxides according to Vogel (23) before using. Each extract was dried on a rotary evaporator.

Test for Antioxidant Activity

A 10 mL portion of each fraction was taken to test for antioxidant activity according to the method of Bishov (18). The ethyl acetate fraction showed the greatest activity and also gave a positive test when tested for the presence of phenols. This fraction was used for further fractionation. This ethyl acetate fraction accounts for 6.32% of the total sample.

Thin-Layer Chromatography

Using a precoated polyamide flexible sheet for thin layer chromatography (24,25), five microliters of a 10 mg/mL solution of the ethyl acetate fraction were spotted and developed in a solution of chloroform/methanol 1:1 v/v. One sheet was examined under ultraviolet light and was later sprayed with a 1% solution

- ¹⁸Bishov, S.J., and Henick, A.S., "A Gas Chromatographic Method for Continuous Accelerated Study of O₂ Uptake in Fats, Journal of the American Oil Chemists' Society, 43:477 (1966).
- ²³Vogel, A.I., "Practical Organic Chemistry Including Qualitative Organic Analysis", Third Enlarged Edition, p. 163, John Wiley and Sons, Inc.
- ²⁴Stahl, E., "Thin-Layer Chromatography: A Laboratory Handbook", pgs. 41-44 and 686-706, Springer-Verlag, New York (1969).
- ²⁵Walters, D., "Monitoring Phenolics in Cigarette Smoke Condensate Fractions by Micro-Polyamide Thin-Layer Chromatography", Journal of Analytical Toxicology, 1:218 (1977).

of α, α' -dipyridyl in ethanol followed by 1% ferric chloride in ethanol (26), a phenol-detecting reagent. A spot one inch from the origin turned purple indicating an antioxidant was present. A second sheet was dotted with linoleic acid where spots had been seen in the UV on the first sheet and heated at 65°C overnight. This sheet had a white spot in the same position as the purple spot on the sprayed sheet, another indication of antioxidant activity. This spot did not fluoresce under long-wave UV light, but quenched under the short-wave UV light.

Preparative Thin-Layer Chromatography

Thirty plates were streaked with 50 μ L of a 50 mg/mL sample of the ethyl acetate fraction and run in chloroform/methanol 1:1 v/v. The location of the antioxidant was marked under UV light and then removed from the plate and placed in a glass column connected to a reservoir. Next 125 mL of 2-propanol were percolated through. The eluant was evaporated to dryness in a rotary evaporator and 6.6 mg of sample were recovered. This sample was dissolved in ethanol.

²⁶Barton, G.M., " α, α' -Dipyridyl as a Phenol Detecting Reagent", *Journal of Chromatography*, 20:189 (1965).

RESULTS AND DISCUSSION

An ultraviolet spectrum obtained showed one peak at 2725 μ . A literature search showed propyl gallate at 2750 μ and gallic acid at 2725 μ . The infrared spectra of the sample and gallic acid were comparable. The mass spectrum showed a molecular ion at 170.022 corresponding to $C_7H_6O_5$ which could be gallic acid and a peak at 153 corresponding to a loss of an OH group.

Three thin-layer chromatography sheets were spotted with the sample, propyl gallate, and gallic acid and developed as before. The first plate was sprayed with α, α' -dipyridyl and ferric

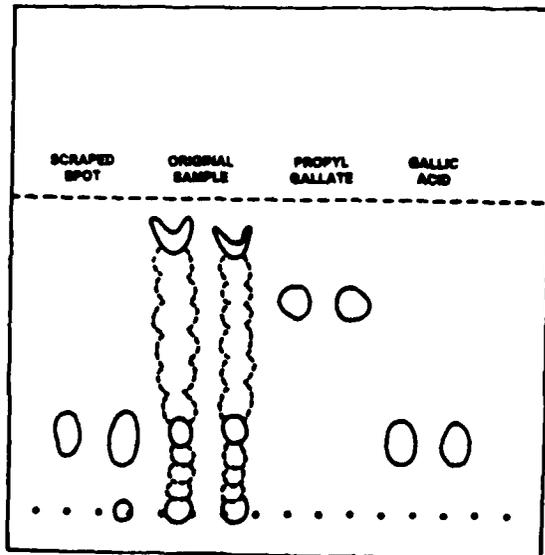


Figure 2. Thin-layer chromatogram of clove antioxidant fraction and standards.

chloride. The gallic acid appeared in the same position as the extracted clove antioxidant. The propyl gallate moved further up the sheet. A second plate was sprayed with 10% linoleic acid in petroleum ether and heated at 65°C overnight. White spots appeared in the same position on the plate where the clove extract was spotted and where gallic acid was spotted. Propyl gallate gave a white spot at about twice the distance.

High Performance Liquid Chromatography

A Waters Liquid Chromatograph equipped with a 7.8 mm x 30 cm μ Bondapak C₁₈ low polarity reverse phase column was used at slow flow rates. Optimum results were obtained by programming the liquid chromatograph for one hour at 1.5 mL/min using curve #3

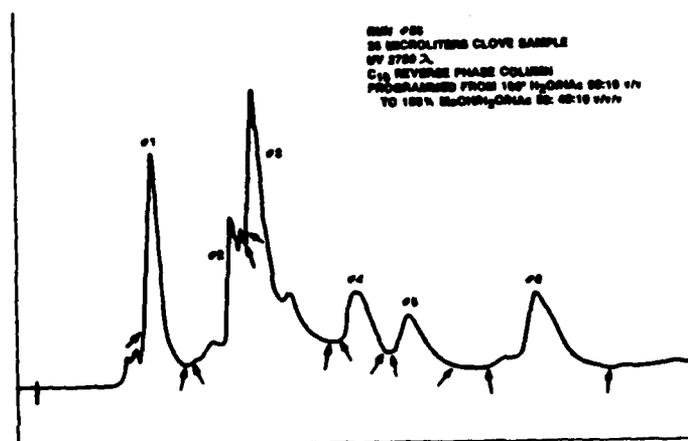


Figure 3. Chromatogram of the ethyl acetate fraction of clove also showing the six areas collected.

and going from 100% Solvent A to 100% Solvent B. Solvent A was water and acetic acid 90:10 v/v and Solvent B was methanol, water, and acetic acid 50:40:10 v/v/v. The ultraviolet detector was set at 2750 γ . The eluted sample from the TLC plates was run as well as the original ethyl acetate fraction to see which peak was the antioxidant. Then propyl gallate, gallic acid and eugenol were run singly in order to compare their elution times with peaks on the original sample.

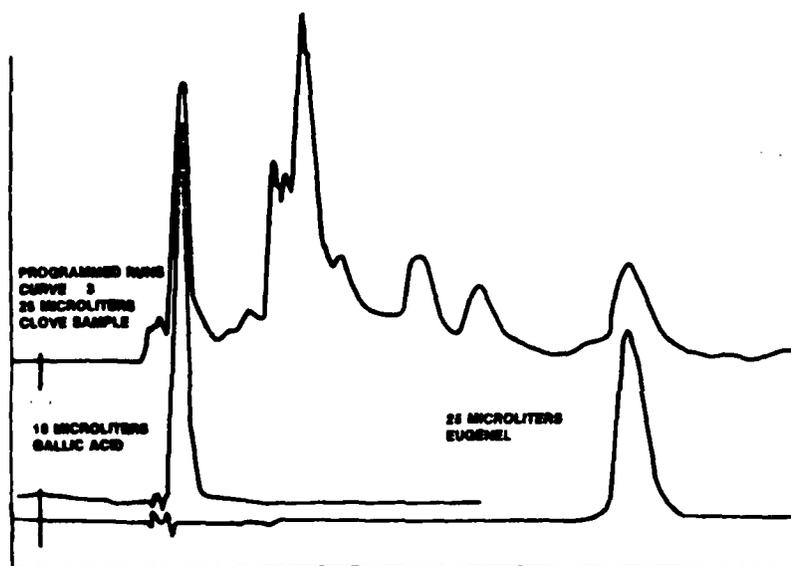


Figure 4. Chromatograms showing the position of gallic acid and eugenol superimposed upon the clove pattern.

The ethyl acetate fraction was spiked with 5 μ L gallic acid, 20 mg/mL in methanol and 5 μ L eugenol, 20 mg/mL in methanol and run. The tentatively identified peaks coincided with the standards and identification was thus confirmed. By HPLC, the amount of gallic acid was determined to be 1.26 g per 100 g clove, and that of eugenol to be 3.03 g per 100 g clove.

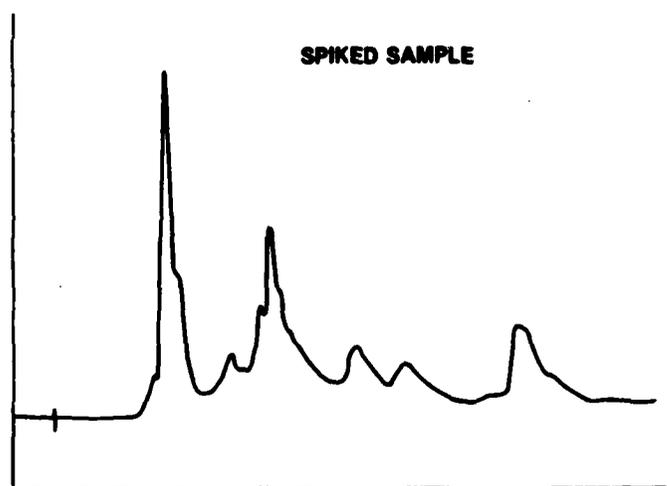


Figure 5. Chromatogram showing the clove fraction that had been spiked with gallic acid and eugenol.

CONCLUSIONS

Besides eugenol, a well known component of clove, gallic acid, a known phenolic antioxidant, has been identified for the first time as one of the natural antioxidants in clove.

This document reports research undertaken at the US Army Natick Research and Development Command and has been assigned No. NATICK/TR-84/035 in the series of reports approved for publication.

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