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PROGRAM AND ABSTRACTS OF PAPERS CITRUS RESEARCH CONFERENCE

December 9, 1963

Fruit and Vegetable Chemistry Laboratory
263 South Chester Avenue
Pasadena, California

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FOREWORD

This Citrus Research Conference is being held to bring to members of the citrus and allied industries in Southern California and Arizona the latest results of research on the chemistry, pharmacology, and technology of citrus fruits and their products carried on by the Utilization Research and Development Divisions of the Agricultural Research Service, U. S. Department of Agriculture. The following Divisions are participating in this year's conference.

Western Utilization Research and Development Division:
Western Regional Research Laboratory (Division
headquarters), 800 Buchanan Street, Albany 10, Calif

Fruit and Vegetable Chemistry Laboratory, 263 South
Chester Avenue, Pasadena, Calif.

Southern Utilization Research and Development Division:
U. S. Fruit and Vegetable Products Laboratory,
600 Avenue S, N. W., Winter Haven, Florida

U. S. Fruit and Vegetable Products Laboratory,
509 West Fourth Street, Weslaco, Texas

PROGRAM
CITRUS RESEARCH CONFERENCE
Monday, December 9, 1963

Abstract
on page

9:30 A. M.

M. J. Copley, Director, Western Utilization Research
and Development Division, Albany, Calif. - Presiding

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LUNCH

1:30 P. M.

E. A. Beavens, In Charge, Fruit and Vegetable Chemistry
Laboratory, Pasadena, California - Presiding

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RECENT ADVANCES IN THE ANALYSIS OF ORANGE AND GRAPEFRUIT ESSENTIAL OILS

G. L. K. Hunter and William B. Brogden, Jr.
Southern Utilization Research and Development Division
Fruit and Vegetable Products Laboratory, Winter Haven, Florida

The phenomenon of flavor is presently receiving a great deal of attention from scientists. The sensation is due to the properties of various chemical components, and it appears that even components occurring in very small concentrations can have a profound effect on flavor. If flavor is to be properly evaluated, methods must be devised for separating and identifying small amounts of material.

With the advent of gas chromatography half of this difficult problem has been overcome, i. e., the microseparation of gross mixtures. It now appears that it is possible to identify these submicrogram quantities as rapidly as they are eluted from a gas chromatogram by use of a Time-of-Flight mass spectrometer as the detector. While much can be deduced from the cracking pattern, true identification can best be obtained by comparison of spectra with those of known chemical constituents. It was therefore necessary to investigate many essential oils in order to obtain authentic samples for purposes of cataloging reference spectra.

This method has been successfully applied to the elucidation of the sesquiterpenes in both grapefruit and orange cold-pressed oil. In order to simplify the analysis, the oils were stripped of low boiling materials by passing the whole oil through a molecular still at reduced pressure. The residual fraction was recycled through the same still, again at reduced pressure, in order to remove the non-volatile substances, waxes, and higher molecular weight materials. The oxygenated compounds in the distillate containing the sesquiterpene hydrocarbons were removed by column chromatography using basic alumina. The eluants containing the sesquiterpene hydrocarbons were further separated by gas chromatography. The eluted fractions were led into the Time-of-Flight mass spectrometer for identification. These fractions were also trapped for further analyses by infrared spectroscopy and chemical means.

As a result of the short contact time in the molecular still, the various fractions appeared to have suffered no decomposition. Comparison of the gas chromatographic curves before and after removal of the oxygenated materials further indicated that the sesquiterpenes suffered no apparent decomposition or isomerization on the column of basic alumina; i. e., they were all present in the same relative concentrations in both chromatograms.

Eight sesquiterpenes have been identified in grapefruit oil and nine in orange oil. Also, the structures of ylangene and two new sesquiterpenes have been postulated.

THE SESQUITERPENES OF LEMON OIL

W. D. MacLeod and Nelida Buigues

Western Utilization Research and Development Division
Fruit and Vegetable Chemistry Laboratory, Pasadena, Calif.

Composition studies on cold-pressed citrus oils have greatly advanced in recent years through the use of gas chromatography on the volatile portion of the oil. Composition of the non-volatile portion of citrus oils also has been worked on extensively, particularly the crystalline solids. As a result of these studies approximately 90 percent of the weight of lemon oil has been identified and assayed. Among the remaining portion still being studied is an interesting family of compounds, the sesquiterpenes. These compounds contain fifteen carbon atoms and are related biogenetically with the more familiar terpenes of citrus oils such as limonene and citral.

Although the sesquiterpene hydrocarbons and oxygenated hydrocarbons comprise only a few percent of the weight of cold-pressed citrus oils it is generally believed that they make a significant contribution to the flavor of some oils.

Initial studies on the chemistry of lemon oil sesquiterpenes have been directed toward the unsaturated hydrocarbons. Cold-pressed oil was folded under partial vacuum to about one-fourth its original volume in order to remove most of the volatile terpene hydrocarbons. Passage of the folded oil through a column of chromatographic alumina in petroleum ether separated the unsaturated hydrocarbons from the oxygenated compounds. There was no evidence that the silicic acid or the basic, neutral, or acid-washed alumina affected the terpene or sesquiterpene hydrocarbons. However, certain brands of silica gel caused considerable deterioration of the sesquiterpenes. Basic alumina should be avoided if the oxygenated fraction is desired subsequently without ester saponification.

After removal of the oxygenated compounds the hydrocarbon fraction was then distilled in a vacuum fractional distillation column to separate the sesquiterpenes, boiling range 70°-90° C at 5 mm. Hg, from the remaining more volatile compounds and the less volatile waxes.

Examination of the sesquiterpene fraction by analytical gas chromatography revealed the presence of eight or more hydrocarbon compounds in the sesquiterpene region. The whole fraction was preparatively gas chromatographed on a 3/8-in. by 10-ft. column packed with 60- to 80-mesh Anakrom ABS impregnated with 20 percent carbowax 20 M. Optimum temperature for good peak resolution and reasonable retention time fell

between 170° and 180° C. The main fractions were repetitively rechromatographed for highest purity. Generally, a lower capacity analytical column, 1/4-in. diameter, was necessary in the final step.

The three major sesquiterpenes in lemon oil were isolated in pure form and identified as β -bisabolene, α -bergamotene and caryophyllene by comparison of their IR spectra and gas chromatographic retention times with those of known compounds.

Preparative gas chromatography is being attempted with those sesquiterpenes present in lesser amounts. Repeated high temperature gas chromatography on compounds potentially sensitive to such treatment necessarily calls for caution in the interpretation of results. Currently, new methods for separating and distinguishing the unsaturated hydrocarbons are being investigated.

STUDIES ON ORANGE FLAVOR

William L. Stanley

Western Utilization Research and Development Division
Western Regional Research Laboratory, Albany, California

The process of removing water from fruit juices in preparing concentrates and powders unavoidably also results in concurrent removal of important volatile flavors. In commercial orange juice concentrates this loss is partially compensated by adding back a small amount of fresh juice or peel oil. Studies on flavor in the Fruit Laboratory at the Western Regional Research Laboratory in Albany are directed toward methods of recovery and reincorporation of the fresh orange juice flavor into juice powders. These studies include investigating the optimum conditions for stripping flavoring components, extraction procedures, investigation of commercial byproduct fractions for flavorings, chemical studies of the components responsible for flavor and taste-panel evaluations. Stripping at moderately high temperatures appears to require removal of less water with volatiles (better efficiency), provided residence times are not too long. Important flavoring material was recovered from oil from orange juice concentrate condensate. Ether extracts of trapped condensate were investigated with capillary gas-liquid chromatography and fast-scan mass spectrometry in combination. Analytical data from this system and fractions obtained from orange oils are presented and discussed.

RECENT DEVELOPMENTS IN THE CHEMISTRY OF CITRUS BITTER PRINCIPLES

David L. Dreyer, Jr.

Western Utilization Research and Development Division
Fruit and Vegetable Chemistry Laboratory, Pasadena, Calif.

As part of a comprehensive study on the bitter principles in Navel oranges Emerson, in 1947, reported the isolation of small amounts of a material, m. p. 315°, which he called substance X. Kefford and Chandler in Australia later reported the isolation of a similar material from citrus seeds which they called limonexic acid. They also showed that this substance could be prepared synthetically from limonin. Later, Arigoni *et al.* reported an improved method of preparation for limonexic acid and suggested a reasonable structural relationship with limonin. Of the two structural possibilities suggested for limonexic acid, that of a β -substituted but-3-enolide now has been shown to be correct on the basis of nuclear magnetic resonance measurements. The NMR spectra of a number of other limonin derivatives also have been made in order to interpret the spectrum of limonexic acid. If limonexic acid is a true metabolite of limonin in citrus then this suggests a rational debittering route which may occur as oranges mature.

A number of limonin derivatives have been prepared and their bitterness determined as follows:

Limonin	bitter
Obacunonic acid	bitter
Limonexic acid	non-bitter
Limonilic acid	non-bitter
Limonin oxime	bitter
Limonin diosphenol	bitter
Citrolin	non-bitter
Desoxylimonin	non-bitter
Limonol	non-bitter
Epimimonol	non-bitter
Hexahydrolimoninic acid	non-bitter
Tetrahydrolimonin	bitter
Obacunone	?
Nomilin	bitter

Thus on the basis of a limited number of derivatives there is about a 50% chance of converting limonin to a non-bitter derivative in any given reaction. On the other hand, the results are sufficiently varied so that no clear-cut generalizations with regard to structure vs. bitterness can be made.

Thin-layer chromatography has been found most useful for monitoring citrus bitter principles. Silicic acid proved to be superior to alumina in this technique. The spots were developed by spraying with para-dimethylaminobenzaldehyde followed by treatment with HCl gas. This gives reddish pink spots. With thin-layer chromatography, which is a sensitive qualitative test, only limonin could be detected in bitter Navel orange concentrate. Similarly, extracts of various citrus seeds, after removal of most of the limonin by crystallization, have been investigated for the presence of bitter principles. Extracts of Valencia orange seeds showed, in addition to obacunone and nomilin, a "bitter principle spot" more polar than limonin. Extracts of grapefruit seeds showed a new "bitter principle spot" between obacunone and limonin and another one more polar than limonin. Using IR and melting points, limonin was shown to be present in extracts of pummelo seeds. Thin-layer chromatography of these extracts indicated the presence of small amounts of obacunone plus a new bitter substance more polar than limonin and probably identical with the new substances shown to be in the extracts of grapefruit and orange seeds.

Recently, several new bitter substances have been reported in the literature which are closely related biogenetically to limonin. Among these are flindissol, anthothocol, evodol, and nimbin. The botanical and biogenetic relationships among these compounds will be discussed.

RECENT STUDIES ON THE O- AND C-GLYCOSIDES OF CITRUS

Robert M. Horowitz, Bruno Gentili, and Elli S. Hand
Western Utilization Research and Development Division
Fruit and Vegetable Chemistry Laboratory, Pasadena, Calif.

The known flavanone glycosides of citrus fruits contain either of two isomeric disaccharides, rutinose or neohesperidose. Rutinose is 6-O- α -L-rhamnopyranosyl-D-glucopyranose, while neohesperidose has been shown recently to be 2-O- α -L-rhamnopyranosyl-D-glucopyranose. Glycosides that contain neohesperidose are often either intensely bitter or intensely sweet, while the corresponding glycosides that contain rutinose are invariably tasteless.

Neohesperidose has been obtained as the free disaccharide by both degradative and synthetic procedures. As reported previously, the degradative procedure involved ozonolysis of either naringin or neohesperidin, followed by charcoal chromatography of the liberated neohesperidose. The synthetic procedure involved the condensation of 1, 3, 4, 6-tetra-O-acetyl- α -D-glucopyranose with 2, 3, 4-tri-O-benzoyl- α -L-rhamnopyranosyl bromide in the presence of mercuric salts and acetonitrile. The product was deacylated and then reacetylated to give 2-O- α -L-rhamnopyranosyl- β -D-glucopyranose hepta-acetate. This was identical with the β -neohesperidose hepta-acetate obtained by the degradative procedure. In addition to confirming the structure of neohesperidose, the synthesis should make it possible to prepare carbon-14 labeled glycosides which can be used in studying metabolism and mechanisms of natural debittering.

The transformation of certain of the bitter citrus flavanones to their corresponding dihydrochalcones results in the formation of intensely sweet compounds. Continuing studies have added further support to the hypothesis that the presence of neohesperidose in the A-ring and a free hydroxyl group in the B-ring is necessary for intense sweetness. There is much interest at the present time in synthetic sweeteners, since the consumption of these compounds is increasing rapidly.

It has become apparent in recent years that the flavone C-glycosides are among the most widely distributed flavonoids in plants. Vitexin, a key compound in this group, has been isolated from orange peel and a number of closely related flavones have been isolated from other citrus fruits. The application of nuclear magnetic resonance measurements has shown that vitexin does not contain a glycofuranose side chain, as was previously thought. Instead, chemical and NMR studies have shown that vitexin is apigenin 8-C- β -D-glucopyranoside. These results will be discussed briefly.

FURTHER STUDIES ON THE METABOLIC DEHYDROXYLATION OF CATECHOL COMPOUNDS

A. N. Booth

Western Utilization Research and Development Division
Western Regional Research Laboratory, Albany, California

In earlier work concerned with the metabolism of citrus flavonoids such as hesperidin, diosmin, and eriodictyol we discovered the unusual reaction involving dehydroxylation. The removal of a hydroxyl group from a benzene ring (dehydroxylation) was also observed when rats were fed chlorogenic acid, caffeic acid, and DOPA (dihydroxyphenylalanine). Evidence for the dehydroxylation reaction was based upon the detection of a monohydroxyphenolic acid in the urine following the ingestion of any of the dihydroxyphenolic compounds mentioned above. This reaction was believed to be endogenous (animal tissue enzyme dependent) until quite recently when it was reported that dehydroxylation could be prevented if an antibiotic such as neomycin was fed. These results, of course, suggested that dehydroxylation was dependent upon intestinal microorganisms. The results of experiments designed to investigate the role of intestinal organisms in the dehydroxylation reaction may be summarized as follows:

1. Caffeic (3, 4-dihydroxycinnamic) acid was used as the substrate for studying the formation of m-hydroxyphenylpropionic acid during the incubation.
2. The occurrence of dehydroxylating activity was found to be wide-spread, and included such sources as rat caecal, colon and fecal contents, human excreta, and sheep rumen fluid.
3. Various factors influencing the dehydroxylation reaction were examined including pH, heat stability, oxygen tension, and antibiotics.
4. Work on the isolation of a specific intestinal anaerobe possessing dehydroxylating activity has not been completed.

FOAM-MAT DRYING OF ORANGE JUICE

A. I. Morgan, Jr.

Western Utilization Research and Development Division
Western Regional Research Laboratory, Albany, California

A new 12-minute color sound motion picture shows an adaptation of foam-mat drying to orange juice. The result of various changes is the trouble-free production of orange powder by foam-mat drying to a final moisture sufficiently low that the product can be packaged without a desiccant. This powder is now also sufficiently dense that a container can hold 71 percent more solids as powder than as orange concentrate.

Adding a wiped film cooler to the foaming equipment has produced a finer bodied foam before drying. The result of this is to permit the production directly on the foam-mat dryer of 1.0 to 1.1 percent moisture product without a scorched flavor. This is sufficiently dry for storage without secondary or in-package drying.

A method of pressing the dry orange powder in the nip of warm rolls has been developed. This results in a ribbon which breaks up to a powder having a bulk density of 0.85 gram/ml. A few hundredths of 1 percent monoglyceride release agent used on the rolls appears in the product. This powder is practically free of entrapped gas. It does not give a sudsy or milky appearance to the reconstituted juice. The dry powder itself shows a deeper color by reason of its compaction. Reconstitution remains rapid, particularly so since the dense product sinks in water.

THE SOURCE OF PRECURSORS OF CAROTENES IN COLORED GRAPEFRUIT

I. FIXATION OF CARBON DIOXIDE

Albert E. Purcell

Southern Utilization Research and Development Division
U. S. Fruit and Vegetable Products Laboratory, Weslaco, Texas

Several years ago, Purcell and Stephens demonstrated by reciprocal grafting of red and white grapefruit that color development was not dependent upon the tree on which the fruit grew. Their experiment did not define whether or not specific precursors were furnished to the fruit by the tree. Experiments have been conducted with radioactive CO_2 to determine this.

The following points appear to have been established:

- (1) By photosynthesis the fruit fixes 10 times more CO_2 into itself in 4 hours than is translocated into it from adjacent leaves in 48 hours.
- (2) CO_2 fixed by the leaves moves into carpels more rapidly than into the peel. When fruit is exposed more CO_2 is found in the peel than in the carpels. During photofixation, label in the carpels of exposed fruit appears to be due to translocation of fixed CO_2 rather than diffusion of free CO_2 . In the dark diffusion becomes more important.
- (3) Dark fixation of CO_2 by the peel is less than one-tenth that of photofixation.
- (4) The relative amounts of label into the non-saponifiable fraction and carotenes of the carpels are essentially the same whether label is supplied via leaves or peel.
- (5) The total amount of label and ratio of label in the various fractions of the carpels are about the same when fruit is in the light and in the dark.

COMPARISON OF THE CAROTENOIDS OF LEMON PEEL, PULP AND FROZEN CONCENTRATE

Henry Yokoyama and Carl E. Vandercook
Western Utilization Research and Development Division
Fruit and Vegetable Chemistry Laboratory, Pasadena, Calif.

At the last citrus research conference we reported on the extraordinary complexity of the carotenoid mixture present in lemon pulp. Additional data have been accumulated on the nature of the carotenoids in lemon peel and in frozen lemon concentrate. Results showed that the carotenoids are present in these products in equally complex mixtures,

Demonstration of the complexity of the carotenoid mixtures was achieved by a preliminary fractionation by partition chromatography followed by adsorption chromatography. The saponified carotenoids which were extracted from the peel and concentrate were separated into three fractions on a column of silica gel saturated with methanol. In turn, these fractions were further separated into individual components on columns of MgO and Microcel C (calcium silicate).

More recently, a comparison was made of the carotenoids from lemons picked at the tree-ripened, yellow stage. Both peel and peeled fruit (pulp) were investigated. The frozen lemon concentrate was obtained from commercial sources. In the extraction of the lemon juice in the preparation of these concentrates some peel oil, which also contains carotenoids, was incorporated in the juice.

Qualitatively, the carotenoid mixture of the peel was similar to that previously found in the pulp. Quantitatively, the carotenoids of the peel and pulp differ chiefly in that the former contained higher percentages of violaxanthin and zeta-carotene and a smaller percentage of cryptoxanthin. Two constituents not found in the peel but present in the pulp were tentatively identified as α -carotene-like and hydroxy- α -carotene-like compounds.

A very striking change was found to have taken place in the carotenoids of frozen lemon concentrate. β -Carotene was observed as the major component in the mixture. None of the carotenoid epoxides was detected, but as was found in the case of lemon pulp the corresponding isomeric furanoxides were found in the concentrate in larger proportions than in the peel.

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AND PATENTS*

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Fruit and Vegetable Chemistry Laboratory
263 South Chester Avenue, Pasadena, California

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U. S. Fruit and Vegetable Products Laboratory
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