OCCURRENCE OF A BITTER PRINCIPLE IN CARROTS

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Arleigh Russell Dodson

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OCCURRENCE OF A BITTER PRINCIPLE IN CARROTS

By

Arleigh Russell Dodson

AN ABSTRACT

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Carrots frequently develop a bitter flavor in storage which has become a serious problem in the canning industry. The purpose of this investigation was to isolate and identify the compound, or compounds, responsible for bitterness.

A bitter principle of carrots was detected qualitatively by paper partition chromatography.

A crystalline, bitter compound, which melts at 77°C. and fluoresces in ultraviolet light, was isolated from bitter carrots. The empirical formula was established as $C_{14}H_{15}O_5$. Qualitative chemical analyses indicated the presence of a free phenolic hydroxyl group which could be methylated with diazomethane, an alkoxyl group, and an ester linkage. The presence of one methoxyl group was substantiated by quantitative alkoxyl determination. The infrared spectrum of the bitter compound suggests that the molecule contains a conjugate-chelate carbonyl; while the ultraviolet spectrum indicates ortho, para-substitution on the benzene ring containing the ester carbonyl.

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INTRODUCTION

INTRODUCTION

Bitterness in carrots is a serious problem in some of our vegetable-producing states and is annually causing a considerable loss to the industry. It is hoped that the development of a hybrid will lead to a variety of consistent quality and uniformity in size, color, and flavor. At present, size, color, and flavor are serious problems of the carrot industry.

Recently a flavor has developed, which appears to be associated with the storage of carrots prior to processing. It has been estimated that ten percent of the annual carrot crop is lost because of this off-flavor which has been described as "bitter," "quinine-like," "soapy bitterness," "alum-like," and "spicy and bitter" (1).

Carrots do not have the characteristic, bitter flavor when harvested, but the flavor develops in common storage at 40-45°F. or in cold storage at 32°F. Carrots are the only common, commercial vegetable which develops bitterness in storage. Other commercial vegetables may develop off-flavors, but none of these has been characterized as bitter. Bitterness in carrots is a serious storage problem and primarily concerns the processing industry.

To obtain a better understanding of the factors involved in the development and control of bitter flavors in carrots, the isolation and identification of the compound, or compounds, responsible for the bitter flavor of carrots have been undertaken.

HISTORICAL

HISTORICAL

There are few reports in the literature concerning the off-flavors of carrots. Yamaguchi, Howard, and McNelly (1) compared bitter and non-bitter carrots and noted no great differences in total sugars, starch, protein, calcium, iron, phosphorus, vitamin C, vitamin B_1, vitamin B_2, niacin, or β -carotene; however, bitter carrots showed a much lower level of α -carotene than non-bitter carrots. These observations led to the postulation that bitterness may be caused by metabolic products of the carotenoids; this has not been confirmed, and no further work has been reported. Newcombe and Alderman (2) found that carrots grown in muck soils developed a rancid taste more readily in storage than carrots from upland soils. "Aster yellows," a virus disease which may infect carrots, causes an astringent off-flavor (3), but an organoleptic panel can distinguish easily bitter carrots from carrots infected with the virus (1). Atkin and Sayre(4,5) described the bitter flavor of carrots and some of the probable causative factors on the basis of work carried out during the years 1953 and 1954. However, the growing seasons in these two years were quite different, and this work can be considered only as preliminary. The years 1955 and 1956 were very poor growing seasons in New York, causing Atkin and Sayre to abandon their research (5); the carrots were of poor quality and could not survive a storage experiment.

Although a bitter compound had not been isolated from the root

of the carrot, Pictet and Court (6) have isolated and identified two alkaloids, pyrrolidine and daucine, which are present in carrot tops.

Gizycki and Hermanns (7), while repeating the work of Pictet and Court, isolated a compound thought to be a bitter glycoside but which they were unable to characterize. Reeb (8) isolated a bitter glycoside from carrot seeds. Sorm, Zaoral, Arient, Pliva, and Herout (9) have identified carotol, α-pinene, β-pinene, p-cymene, carvone, geranyl acetate, β-caryophllene, bergamotene, bisabolene, other diterpenic hydrocarbons, and daucol in the volatile oil of carrots. Most of these terpenes have a bitter flavor.

Two empirical methods have been developed for measuring the bitterness of carrots, but in neither case has the compound been isolated or identified. Phillips (10) blended a carrot sample with acetone (1 ml./g.) for five to ten minutes. The acetone was decanted and washed with 100 ml. portions of Skellysolve "B"[1] until the Skellysolve layer was colorless; usually three or four portions of Skellysolve were sufficient. The solvent extracts were combined, concentrated to 25-40 ml. by evaporation, and chromatographed on a column of magnesium oxide:Celite (2:1 by weight). After developing the column with 500 ml. of Skellysolve "B", the top 3 to 6 mm. of adsorbent were removed and eluted with spectrograde methanol.[2] The eluate was diluted quantitatively to 50 ml. and its ultraviolet spectrum determined with a Beckman Spectrophotometer Model DU using

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[1] Skellysolve "B" purchased from Skelly Oil Company, Chicago, Illinois.

[2] Methanol (Spectro Grade) S 467 purchased from Distillation Products Industries, Eastman Organic Chemicals Department, Rochester 3, New York.

one centimeter cells and spectrograde methanol as a compensating solvent. Readings were taken at 248 mμ, 268 mμ, and 290 mμ. These data were plotted (wave length (mμ) versus percentage transmittance), and readings which exhibited a maximum absorption at 268 mμ indicated the bitter substance to be present. The concentration of the latter compound could be estimated by the length of the vertical line drawn from the point at 268 mμ to the straight line connecting the points at 248 mμ and 290 mμ.

Sondheimer, Phillips, and Atkin (11) have described also a quantitative method for determination of bitterness based upon the characteristic ultraviolet absorption spectrum of bitter carrot extracts which eliminates the time consuming chromatographic step of the Phillips method. Thus, carrots were blended for five minutes to a workable consistancy, and a 5 g. sample was weighed into a 60 ml. ground-glass stoppered bottle containing 40 ml. of spectrograde Skellysolve "B"[1] or spectrograde cyclohexane.[2] The bottle was shaken, the supernatant liquid decanted, and readings were taken at 290 mμ, 265 mμ, and 240 mμ with a Beckman Spectrophotometer Model DU or DK2 using one centimeter cells. The spectrophotometric data were expressed as "height of the 265 mμ peak" and appear to have been

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[1] Prepared by the method of H. H. Graff, Purification of Solvents for Absorption Spectroscopy, Anal. Chem., 16, 556 (1944).

[2] Cyclohexane (Spectro Grade) S 702 purchased from Distillation Products Industries, Eastman Organic Chemicals, Rochester 3, New York.

calculated by the formula:

$$\frac{\left[A\ 265\ m\mu - \left(\frac{A\ 290\ m\mu + A\ 240\ m\mu}{2} \right) \right] \text{ ml. solvent}}{\text{g. sample}}$$

where A represents absorbance. Excellent correlation was found between "height of the 265 mμ peak" and the actual bitterness of carrots as judged by an organoleptic panel.

EXPERIMENTAL

EXPERIMENTAL

The experimental work consisted of the development of a new method for detection of the bitter principle and the isolation and characterization of this principle.

All solvents were C.P. solvents unless otherwise noted. Melting points were determined on a Fisher-Johns block and were not corrected.

PART I. DETECTION

A rapid, qualitative method for the detection of the bitter principle was developed using paper partition chromatography (12). Bitter carrots were extracted with acetone according to Phillips' procedure (10) and the acetone extracts applied to Whatman #1 filter paper (2.5 x 25 cm.). All chromatograms were developed by ascending technique and were not equilibrated. The Rf values were easily detected by their bright, blue-white fluorescence in ultraviolet light. Table I lists the Rf values of the acetone extract of bitter carrots chromatographed with various solvent systems.

TABLE I

Rf VALUES OF THE ACETONE EXTRACT OF BITTER CARROTS

Solvent	Rf Values
Water	0.44
Phenol saturated with water	0.97
Acetic acid (15%)	0.74
Heptane:1-butanol:water (29:14:57 by volume)	0.58
Ethyl acetate:ammonia (2N) (1:1 by volume)	0.55

To ascertain whether the blue-white, fluorescing spots were actually the compound being measured by the method of Phillips, the spots from the chromatograms were eluted with spectrograde methanol. The ultraviolet absorption spectrum of the methanol eluate and the spectrum of the methanol eluate of the Phillips method were identical. The Skellysolve "B" eluate of the blue-white, fluorescing spot gave an ultraviolet absorption spectrum identical with the spectrum of the Skellysolve "B" extract obtained by the procedure of Sondheimer, et al. Therefore, paper partition chromatography and/or ultraviolet absorption spectra were used for the subsequent isolation and identification work.

PART II. ISOLATION AND IDENTIFICATION

Fourteen bushels of bitter carrots from the 1954 carrot crop were dried in a forced-draft oven for three hours at 60°C. and ground in a Wiley mill to pass a 2 mm. sieve. Twenty pounds of the ground material was extracted in a large Soxhlet unit with acetone for eight hours. The acetone solution was concentrated under reduced pressure (water aspirator) until crystals began to separate from the solution. The colorless crystals were collected by filtration; concentration was continued to a tarry mass. Further attempts to isolate additional crystalline material from the tarry mass failed so the residue was discarded. One hundred milligrams of crystalline material was obtained which possessed the characteristic flavor of bitter carrots and which fluoresced brightly with a blue-white glow in ultraviolet light. Recrystallization from aqueous methanol gave colorless platelets, m. p. 77°C. The compound was dried over phos-

phorus pentoxide *in vacuo* at 25°C. The crystalline bitter compound had the same Rf value in each of the solvent systems listed in Table I as the acetone extract of bitter carrots prepared according to Phillips' procedure (10) or the Skellysolve "B" extract obtained by the method of Sondheimer, *et al.* (11).

In 1956, using carrots from the 1955 crop and the same isolation procedure, an additional two hundred milligrams of the crystalline, bitter compound was obtained. No bitter carrots were available in 1956 because of the poor growing season.

A sample of the bitter compound was fused with sodium and subjected to elemental analysis; halogens, nitrogen, and sulfur were absent (13). A nitrogen analysis of 0.00% by Dumas' procedure further substantiated the elemental analysis[1]. The results of qualitative tests (14) on the bitter compound are summarized below.

Test	Result	Conclusion
Molisch's	Colorless solution	No carbohydrate present
Millon's	Red solution	Aromatic ether or phenol with unsubstituted *ortho*-position
Liebermann's	Green solution	Aromatic ether or phenol with unsubstituted *para*-position
Azobenzenephenylhydrazine sulfonic acid	Orange solution	No aldehyde or ketone present
Sodium cobaltinitrite	Yellow solution	Phenolic hydroxyl with unsubstituted *ortho*-position
Starch-iodide	Colorless solution	No acid present

[1] The microanalyses were performed by T. L. Rebstock, Department of Agricultural Chemistry, Michigan State University, East Lansing, Michigan.

Quantitative analysis showed:[1]

Carbon	64.03%	Hydrogen	5.87%
	63.84%		5.88%
Average	63.94%	Average	5.88%

These data indicate that the bitter compound contains a large percentage of oxygen. Huffman Microanalytical Laboratories, Wheatridge, Colorado found the molecular weight of the bitter compound to be 268 (Rast camphor method).

The percentage of alkoxyl in the bitter compound was determined by the volumetric procedure of Vieboch and Brecher (15). The alkoxyl group was cleaved with hydriodic acid, and the resulting alkyl iodide (methyl or ethyl) was oxidized by bromine to iodic acid. Treatment of the iodic acid with excess potassium iodide in acid solution gave iodine, which was titrated with sodium thiosulfate.

Weight, mg.	Volume (0.0200 N $Na_2S_2O_3$), ml.	Blank, ml.	Methoxyl, %
5.055	7.58	0.15	15.22
5.030	6.73	0.15	13.53
4.534	6.69	0.15	14.92

To determine if further methylation were possible, the bitter compound was treated with diazomethane (16). Sodium hydroxide was added to 5 g. nitrosomethylurea, and the evolved diazomethane was collected in peroxide-free ether. The ethereal solution of the diazomethane was added to 0.2786 g. of the bitter compound dissolved in 10 ml. of methanol. After keeping the reaction mixture at 0°C. for

[1] The microanalyses were performed by T. L. Rebstock, Department of Agricultural Chemistry, Michigan State University, East Lansing, Michigan.

six hours and at 25°C. for an additional 12 hours, fine white needles (0.1666 g.) separated. Recrystallization from methanol, followed by drying over phosphorus pentoxide *in vacuo* at 25°C., gave a compound which melted at 127-8°C.

The findings of the qualitative and quantitative tests of the methyl derivative are summarized below.

Test	Results	Conclusion
Liebermann's	Yellow solution	Aromatic ether or phenol with unsubstituted *para*-position
Sodium cobaltinitrite	Pink solution	No phenolic hydroxyl with unsubstituted *ortho*-position
Ferric chloride (aq.)	Yellow solution	No phenolic hydroxyl
Starch-iodide	Colorless solution	No acid
Hydroxamic acid	Violet-red solution	Ester or lactone

Quantitative analysis:[1]

Carbon	64.86%	Hydrogen	6.08%
	65.25%		6.35%
Average	65.06%	Average	6.22%

Alkoxyl determination:

Sample, mg.	Volume (0.0102 N $Na_2S_2O_3$), ml.	Blank, ml.	Methoxyl, %
5.707	31.31	0.39	28.5
5.677	31.48	0.39	28.8

[1] The microanalyses were performed by T. L. Rebstock, Department of Agricultural Chemistry, Michigan State University, East Lansing, Michigan.

The bitter compound, the methyl derivative, and several model compounds were subjected to infrared spectral analysis. Infrared spectra were determined using chloroform solutions of the compounds, sodium chloride solution cells (0.5 mm.), and a Perkin-Elmer Model 21 Spectrophotometer. Special attention was directed to the carbonyl stretching frequency range of the infrared spectra, since the presence of an ester or lactone was indicated by the qualitative tests. The stretching frequencies associated with the carbonyl group in the bitter compound, methylated derivative, and several model compounds are listed in Table II.

TABLE II

THE INFRARED CARBONYL STRETCHING FREQUENCY OF SEVERAL COMPOUNDS

Compound	$\lambda\mu$	$cm.^{-1}$
Bitter compound	6.00	1667
Methylated derivative	5.85	1709
Methyl 2-hydroxybenzoate	5.95	1681
Methyl 2-methoxybenzoate	5.80	1724
p-Methoxyphenyl 2-hydroxybenzoate	5.90	1695
p-Methoxyphenyl 2-hydroxybenzoate	5.75	1739

The ultraviolet absorption spectra of ethanolic (95%) solutions of the bitter compound, the methyl derivative, and methyl 2-hydroxy-4-methoxybenzoate were determined in one centimeter cells using a Beckman Spectrophotometer Model DK2. Table III gives the wave lengths at which maximum absorption occurs and the specific extinction coefficients (concentration in g./l.) for the B and C bands of these

three compounds.

TABLE III

ULTRAVIOLET ABSORPTION SPECTRA MAXIMA AND SPECIFIC EXTINCTION COEFFICIENTS

Compound	B band max, mμ	K	C band max, mμ	K
Bitter compound	266	72.92	307	29.34
Methyl derivative	262	69.41	297	32.21
Methyl 2-hydroxy-4-methoxybenzoate	260	----	297	----

The ultraviolet spectrum of the bitter compound determined in spectrograde Skellysolve "B" exhibited maxima at 265 mμ and 300 mμ; minima occured at 240 mμ and 278 mμ.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

This study was undertaken to isolate and identify the compound, or compounds, responsible for the bitter flavor of carrots; therefore, it was necessary to establish that the compound isolated was actually the bitter substance. Sondheimer, et al. reported that the Skellysolve "B" extract of bitter carrots showed maximum absorption at 265 mμ while non-bitter carrots did not absorb in this region. Paper partition chromatography of the bitter carrot extracts gave a fluorescent spot with a characteristic Rf value; this spot did not appear during chromatography of non-bitter carrot extracts. Therefore, it was concluded that a compound exhibiting maximum absorption at 265 mμ in Skellysolve "B" and giving a fluorescent spot with a characteristic Rf value upon paper chromatography was either the compound responsible for the bitterness in carrots or an "index" compound present in proportion to the bitter principle.

A fluorescent, crystalline, bitter compound was isolated. A Skellysolve "B" solution of this compound showed maximum absorption at 265 mμ and gave fluorescent spots at Rf values that are in agreement with those of Dodson, Fukui, Ball, Carolus, and Sell, hence the compound isolated is either the bitter principle or an "index" compound.

The carbon and hydrogen analyses suggest an empirical formula $C_{14}H_{15}O_5$ for the bitter compound. The molecular weight, 263, calculated from the empirical formula agrees with the experimentally determined value, 268. Attempts to determine the molecular weight of

the methyl derivative by the Rast method using camphor or exaltone as solvents failed.[1] A molecular weight of 277 has been assumed for the methyl derivative on the basis of the empirical formula suggested by carbon and hydrogen analyses.

On the basis of a molecular weight of 277 for the methyl derivative and the observed molecular weight of 268 for the bitter compound, the former compound contains 28.6% methoxyl while the latter compound contains 14.6% methoxyl. An assumption was made that the alkoxyl determination was for methoxyl, not ethoxyl; this was necessary because the alkoxyl determination of the bitter compound gave values of 14.56% for methoxyl or 21.3% for ethoxyl. These percentages correspond to 1.26 methoxyl or 1.27 ethoxyl per molecule. If the postulate is correct, then the methoxyl content of the bitter compound and its methyl derivative provides further evidence that one hydroxyl group of the bitter compound was methylated; thus the bitter compound contains one methoxyl group while two methoxyl groups are present in the methyl derivative.

Qualitative tests indicated the presence of an ester or lactone in the bitter compound and its methyl derivative; therefore, the regions of the infrared spectra of these compounds associated with the carbonyl group were examined. A four or five-membered lactone carbonyl absorbs at wave numbers greater than 1770 $cm.^{-1}$ (5.65μ) (17), but the bitter compound or its methyl derivative absorb beyond this region. Thus the bitter compound and its derivative must be either esters or six-membered lactones.

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[1] Schwarzkopf Microanalytical Laboratory, 56-19 37th Avenue, Woodside 77, New York.

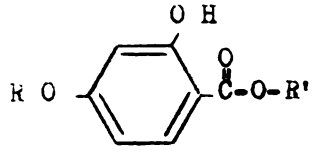
An unconjugated carboxylic ester or six-membered lactone carbonyl absorbs at 1739 $cm.^{-1}$ (5.81μ) (17). Enolized β-diketones or ortho phenolic hydroxyl groups exhibit the bathochromic shift of the carbonyl stretching frequency expected from conjugated chelate systems, e.g., the carbonyl absorption appears near 1667 $cm.^{-1}$ (6.00μ). The infrared spectrum of the bitter compound shows absorption at 1667 $cm.^{-1}$ (6.00μ), therefore it may be concluded that the carbonyl group is located ortho to a phenolic hydroxyl group. Methylation of the phenolic hydroxyl shifts the carbonyl stretching frequency to 1709 $cm.^{-1}$ (5.85μ) suggesting that the carbonyl group is conjugated with a benzene ring.

If the conjugate-chelate carbonyl is in a six-membered lactone, the lactone ring must be attached to the para-position of the benzene ring carrying the carboxyl carbonyl or to a group on the 6-position of the benzene ring. In either case, seven carbons are accounted for; the remaining eight carbons must be attached to the 6-position of the benzene ring. Positive Millon and Liebermann tests preclude placement of substituents in the 3 and 5-positions of the benzene ring. Attachment of six carbons in the 6-position is improbable on the basis of steric factors, hence the possibility of a six-membered lactone has been eliminated, and the bitter compound must contain an ester linkage.

Substituted benzoic acids exhibit three absorption bands in the ultraviolet which have been designated as the A, B, and C bands (18). The frequency of absorption of A is independent of, B is very dependent upon, and C is somewhat dependent upon the nature and position of the substituent groups. Since the bitter compound has maxima

corresponding to the B and C bands at 266 mμ and 307 mμ, respectively, in 95% ethanol, and since an ortho hydroxyl group was indicated by the infrared spectrum, it may be concluded that the bitter compound has ortho, para-substitution on the benzoic acid portion of the molecule. On the basis of elemental analysis and spectral data, the group in the para-position is either an ether or a free hydroxyl group, since an aliphatic group would cause the least bathochromic shift of the B band. Methylation of the bitter compound introduced one methoxyl group ortho to the ester carbonyl, therefore a free hydroxyl group in the para-position appears unlikely. On the basis of these data it may be concluded that the para-position contains an ether group.

The para-position could contain either an aryl or an alkyl ether group; if it is an aryl ether, then the compound is a methyl ester. If the compound is an aryl ester, then the para-position of the benzene ring carries a methoxyl group. Either of these partial structures leaves three hydrogens and one oxygen unassigned. Another possibility exists, i.e. the ester and the ether may be aliphatic with one being a methyl group. The possibilities are summarized by the following partial structural formula:



R or R' = CH_3

R or R' = C_6H_8O

Hydrolysis of the ester and identification of the hydrolysis products should lead to complete elucidation of the structural formula. At present, the quantity of the bitter principle which is available has restricted further work.

SUMMARY

SUMMARY

Paper partition chromatography was used as a qualitative method for the detection of a bitter principle of carrots.

A crystalline, bitter compound, m. p. 77°C., was isolated from carrots and has been assigned the empirical formula $C_{14}H_{18}O_5$. Qualitative chemical analysis indicated the presence of a free phenolic hydroxyl group which could be methylated with diazomethane, an alkoxyl group, and an ester linkage; while a quantitative alkoxyl determination showed that the molecule contains one methoxyl group. Spectral analyses suggested the presence of a conjugated-chelate carbonyl and ortho, para,-substitution on the benzene ring carrying the ester carbonyl.

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