[CONTRIBUTION FROM DIVISION OF FOOD TECHNOLOGY, UNIVERSITY OF CALIFORNIA]

The Chemical Deterioration of Dried Fruit during Storage. III. Chromatographic Separation of Carbonyl Compounds as 2,4-Dinitrophenylhydrazones

BY FLOYD H. STADTMAN

It has been shown that furfurals are produced during the browning of dried apricots,[1] and that the addition of furfural to apricot concentrates increases the rate of browning.[2] These observations indicate a possible role of furfurals as intermediates in browning reactions. To test this hypothesis, apricot concentrate was continuously extracted with ethyl acetate during storage to remove furfurals, if present, as rapidly as they were formed. No browning occurred as long as extraction was continued.[2] This experiment showed that browning intermediates are ethyl acetate extractable. The addition of 2,4-dinitrophenylhydrazine reagent to the extracted material (transferred back to water) resulted in the precipitation of 2,4-dinitrophenylhydrazones, indicating that carbonyl compounds were among the extracted substances. It remained to be proved, however, that furfurals were among the compounds extracted.

In this paper a method is described for separating and identifying 2,4-DNPHs[2a] whereby furfural and hydroxymethylfurfural are shown to be two of the many carbonyl compounds found to be present in the ethyl acetate extract. Fractionation of these carbonyl compounds was accomplished by the chromatographic separation of their DNPH's. An attempt to characterize these derivatives was made by spectroscopic analyses. It has been shown that DNPH's differ in their absorption spectra.[3,4] In many cases, it is possible to identify the individual hydrazones by their absorption spectra alone. There are a number of instances, however, where two or more DNPH's have virtually identical absorption. In such cases the individual compounds can be identified by spectroscopic in conjunction with a chromatographic analysis. For example, the DNPH's of acetone and levulinic acid have almost identical absorption spectra, but when chromatographed together and eluted with 20% benzene in petroleum ether they are easily separated, the levulinic acid DNPH being much more strongly adsorbed. Likewise, the absorption spectra of the DNPH's of furfural, hydroxymethylfurfural and methylfurfural are almost identical, yet Wahhab separated them quite readily by chromatography.[1]

DNPH's are very insoluble in water and are readily precipitated from dilute solutions, yet a few milligrams dissolved in organic solvents produce enough color in solution so they can be readily followed on an adsorption column. Strain[5] points out that a number of substances are suitable as adsorbents for 2,4-dinitrophenylhydrazine derivatives and cites results with the use of talc. Other investigators have used activated alumina,[6,7,8,9] and more recently, a silicic acid–Super-Cel mixture has been used.[10]

Experimental

A number of substances were tested for use as adsorbents. These included talc, calcium carbonate, magnesium sulfate, magnesium oxide, silica gel, 50% mixtures of talc and silica gel, and mixtures of talc and Hy-flo filter cel. Fair success was attained using talc as an adsorbent but results varied greatly depending on the brand and even the lot of material used. Most consistent results were obtained by using powdered magnesium sulfate. Material from several lots of two brands was used.[11a] All gave satisfactory results when powdered to pass a 100-mesh sieve. Columns were 2–3 cm. diameter and 8–10 cm. long. The adsorbent was firmly pressed in place with a tamping rod. Before use, columns were washed with 100–200 ml. of benzene to remove benzene-soluble contaminants, and the benzene remaining on the column after washing was displaced with petroleum ether. The adsorption of DNPHs' on magnesium sulfate varies greatly with the solvent. The degree of adsorption decreases with the solvents in the following order: petroleum ether, benzene, ethyl acetate, alcohol. In changing from one eluant to another, gradual changes in adsorption affinity are effected by using mixtures of the two, starting with a low concentration of the new solvent and increasing it when necessary until the first solvent is eliminated entirely. This procedure insures distinct separations and prevents "running together" or "piling up" of bands due to abrupt solvent changes.

It was found that all commercially prepared solvents (C.P. grade) used as eluants contained small quantities of non-volatile contaminants showing absorption in the region between 300 and 220 mμ. Though small, this absorption is of sufficiently high intensity to prevent exact duplication of results. This source of error was reduced but not completely eliminated by further purification of the solvents until a blank (50 ml. evaporated to dryness and the residue taken up in 10 ml. of 95% ethyl alcohol) showed negligible absorption. To purify the benzene, it was washed with 1 N sulfuric acid then with 1 N sodium hydroxide, and redistilled. Petroleum ether and ethyl acetate were purified by redistillation.

Interfering substances also were found to be present in ordinary filter paper. For this reason, sintered glass filters were used for filtration.

Three to five milligrams of DNPH's precipitated from material extracted from apricot concentrates,[11b] dissolved

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(1) Wahhab, THIS JOURNAL, **70**, 3580 (1948).

(2) Haas, E. R. Stadtman, F. H. Stadtman and Mackinney, *ibid.*, **70**, 3576 (1948).

(2a) Hereafter in this paper 2,4-dinitrophenylhydrazone will be abbreviated DNPH.

(3) Braude and Jones, *J. Chem. Soc.*, 498 (1945).

(4) Roberts and Green, THIS JOURNAL, **68**, 214 (1946).

(5) Strain, *ibid.*, **57**, 758 (1935).

(6) Lucas, Prater and Morris, *ibid.*, **57**, 725 (1935).

(7) Buchman, Schlatter and Reins, *ibid.*, **64**, 2701 (1942).

(8) Jones, Wilkinson and Kerlogue, *J. Chem. Soc.*, 391 (1942).

(9) Penney and Zilva, *Biochem. J.*, **37**, 403 (1943).

(10) Roberts and Green, *Ind. Eng. Chem., Anal. Ed.*, **18**, 335 (1946).

(11a) Lots 17 and 19, Baker and Adamson, General Chemical Company, New York, N. Y., and lot 31846 Baker Chemical Company, Phillipsburg, N. J.

(11b) The method of extraction is described in Paper No. I of this series.[2]

in 20–30 ml. of a 1:1 **benzene–petroleum ether** solution, were adsorbed on a freshly prepared magnesium sulfate column. To maintain an even and rapid rate of elution, suction was applied to the system. It seemed likely that some DNPH's might have such low adsorption affinities from 1:1 mixtures of benzene and petroleum ether that they would be eluted together as the first band.[11c] Therefore, the first band eluate was evaporated to dryness, the residue was dissolved in petroleum ether, and developed with a series of concentrations of benzene in petroleum ether.

Spectral analysis could not be made on the eluate directly since values below 276 mμ could not be determined in the presence of benzene, and it is in the region between 255 and 220 mμ that characteristic absorption for many of the hydrazones occurs. All samples were, therefore, transferred to 95% alcohol for spectroscopic analysis. Each eluted fraction was evaporated to dryness. To remove occluded benzene, the residue was dissolved in 5–10 ml. of ethyl alcohol (95%) and again evaporated to dryness. Samples were finally dissolved in 10 ml. of 95% ethyl alcohol and their spectra determined using a DU Beckman Spectrophotometer.

Since furfural and hydroxymethylfurfural were believed to be in the extract, their DNPH's were prepared for comparison purposes. To obtain pure derivatives, aqueous solutions of completely colorless re-distilled furfural (Eastman Kodak Company) and hydroxymethylfurfural prepared after the method of Haworth and Jones[12] were precipitated upon the addition of saturated solutions of 2,4-dinitrophenylhydrazine in 2 *N* hydrochloric acid. Both 2,4-dinitrophenylhydrazine derivatives were recrystallized from 95% ethyl alcohol. Purity of each furfural derivative was indicated by single band formation when the compound was chromatographed. The furfural derivative melted at 229°, and the hydroxymethylfurfural derivative melted at 199°, *cf.* ref. 1.

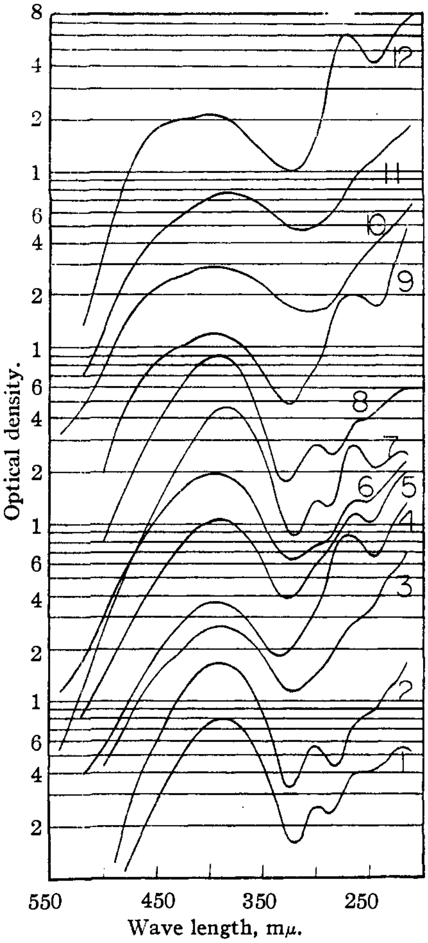

Fig. 1.—Absorption spectra of DNPHs of carbonyl compounds formed in apricot concentrate during storage at 57° (numbered in order of increasing adsorption affinity on magnesium sulfate.).

The absorption spectra of the two compounds were similar with λ_{max} of the principal band absorption at 390 mμ, while λ_{max} for the three secondary bands were 300, 260, and 220 mμ for furfural DNPH and 300, 260, and 218 mμ for the hydroxymethylfurfural derivative. When chromatographed, furfural DNPH exhibits a low adsorption affinity on magnesium sulfate being easily eluted with 20% benzene in petroleum ether, while the hydroxymethylfurfural derivative was much more strongly adsorbed requiring pure benzene for very slow elution, and 20% ethyl acetate in benzene for more rapid elution.

Results

Fifteen separate bands were observed when the "apricot" DNPH mixture was chromatographed. Table I shows the eluant required to remove each band from the column. Of the fifteen bands, twelve were present in sufficient amounts for their absorption spectra to be determined (Fig. 1). Two fractions (nos. 1 and 8) were identified as furfural and hydroxymethylfurfural DNPHs, respectively. Each of the two unknowns had essentially the same absorption spectrum as its corresponding known furfural derivative (Fig. 2). Slight differences in the absorption in the shorter wave lengths (300–210 mμ) were due to the presence of residual ma-

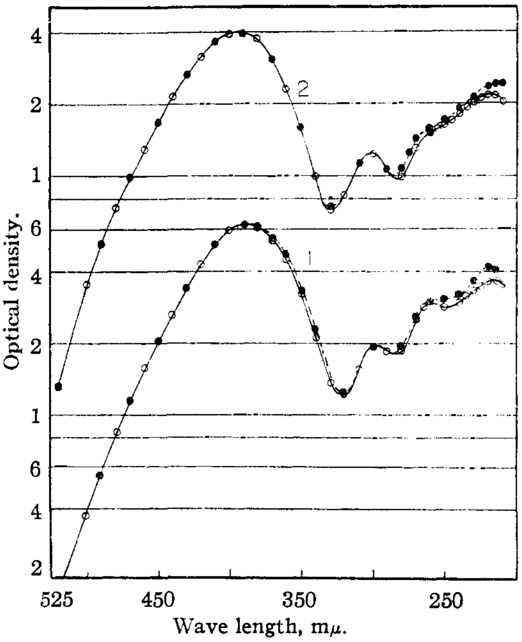

Fig. 2.—Absorption spectra of DNPHs of furfural and hydroxymethylfurfural (open circles, 1 and 2, respectively) superimposed on curves for unknowns (closed circles). (Bands I and VIII, see text.)

[11c] Petroleum ether could not be used as the starting solvent because a number of DNPH's are only slightly soluble in it.

[12] Haworth and Jones, *J. Chem. Soc.*, 667 (1944).

terials in the eluting solvents which were not completely removed by purification techniques used. Final proof of the identity of the unknowns, however, does not come from the spectroscopic data alone, which is of greatest value in enabling one to select the compound or group of compounds to be considered for mixed chromatograms. Fraction I had the same adsorption affinity on magnesium sulfate as the furfural DNPH derivative, while the adsorption characteristics of Fraction VIII agreed with those for the hydroxymethylfurfural derivative (Table I). For final proof of identity, mixed chromatograms were run: Equal amounts of Fraction I and furfural DNPH were adsorbed on magnesium sulfate from petroleum ether and eluted with 20% benzene in petroleum ether; likewise, equal amounts of Fraction VIII and hydroxymethylfurfural DNPH were adsorbed from benzene and eluted very slowly with the same solvent. In both instances no separation was observed. The results of the mixed chromatographs together with the almost identical adsorption spectra are taken as proof of homogeneity in both cases.

TABLE I

COLOR IN ALKALINE ETHANOL WITH RESPECT TO ADSORPTION

	Band	Color in alkaline alcohol soln.	Eluant to remove from column
Increasing Adsorption ↓	1	Red	20% Benzene in petroleum ether
	2	Red	
	3	Red	50% Benzene in petroleum ether
	4	Red	
	5	Red	Benzene
	6	Red	
	7	Red	
	8	Red	20% Ethyl acetate in benzene
	9	Purple	Ethyl acetate
	10	Purple	
	11	Blue	
	12	Blue	Abs. ethanol
	Furfural 2,4-DNPH[a]	Red	20% Benzene in petroleum ether
	Hydroxymethylfurfural 2,4-DNPH	Red	20% Ethyl acetate in benzene

[a] 2,4-Dinitrophenylhydrazone.

Discussion

This work demonstrates that furfural and hydroxymethylfurfural are among the browning intermediates removed by continuous ethyl acetate extraction of apricot concentrate during storage, and it brings to light the fact that not only furfurals, but at least thirteen other carbonyl compounds are also extracted, some or all of which may be involved in browning reactions. The identification of an unknown DNPH by the method described in this paper is not practical unless the number of compounds to be studied for chromatographic comparison can be limited. Elimination of a large number of compounds is possible if the absorption spectrum of the unknowns is virtually identical with the absorption spectrum of a definite known or group of known compounds. Classified absorption data for large numbers of 2,4-DNPH's are very useful. Noteworthy in this connection is the work of Braude and Jones,[3] who studied the absorption spectra of 50 DNPH's. With the exception of the furfural derivative, however, none of them correspond to any of the DNPH's prepared from the apricot sirup extract. Identification of unknowns will be more readily accomplished when absorption spectra for still larger numbers of DNPH's have been studied and classified.

Considering the data presented in this paper collectively, a number of trends were observed which may be of value as aids in identification of individual fractions: (a) for none of the unknowns was λ_{max} for the absorption between 345 and 500 mμ (attributed to the phenylhydrazine half of the molecule) lower than 385 mμ; (b) going from the most weakly adsorbed to the most strongly adsorbed fraction (Fig. 1), there is a gradual shift of λ_{max}, for the "phenylhydrazine" absorption, toward the longer wave lengths; (c) there is a broadening of the phenylhydrazine absorption band, and a decrease in its extinction with respect to the extinction of bands in the shorter wave lengths; (d) there is a progressive change in color from red to blue when ethanol solutions of the unknowns are treated with sodium hydroxide (Table I). It has been shown[3,4] that the position of the absorption band attributed to the phenylhydrazine half of the molecule is dependent upon the extent of conjugated unsaturation, and in a lesser degree upon the amount of alkyl substitution present in the parent carbonyl compound, and general classifications of DNPH's based upon this knowledge have been made. As none of the maxima for the compounds from apricots is below 3850 Å., they would fall into two groups, according to the above mentioned classifications: (a) derivatives of carbonyl compounds having two or more conjugated double bonds,[3] and (b) compounds with derivatives of carbonyl aromatic rings conjugated with double bonds.[4] The furfural derivatives fall in the first group. It should be pointed out, however, that these classifications may not be all inclusive for other characteristics besides the degree of unsaturation present in the parent carbonyl compound can alter the position of the "phenylhydrazine" absorption. For example, λ_{max} increases considerably for bis-DNPH's in the case of α-diketones where conjugation occurs between the two hydrazone systems.[3] None of the fifty compounds studied by Braude and Jones[3] had a greater ϵ_{max} value for absorption between 3000 and 2100 Å. than that for the phenylhydrazine part of the molecule. This characteristic is exhibited, however, by several of the DNPH's prepared from apricot sirup, and suggests the possibility that it is common to a group of substances not represented in studies of known compounds. It is well known that alcoholic DNPH solutions change color from yellow to red or from yellow to blue when they are made alkaline. The resultant color has been used as the basis for colorimetric analytical methods in a number of cases. The results of work reported in this paper indicate a correlation between the color of DNPH's in alkaline ethanol solution and the degree of adsorption on magnesium sulfate. Furthermore, wide "phenylhydrazine adsorption" bands seem to be associated with compounds which give a blue color change in alkaline alcohol. These properties may be characteristic of a particular type of DNPH. The formation of a red color in alkaline ethanol is characteristic of simple aldehyde and ketone dinitrophenylhydrazones, while the formation of blue colors is associated with 2,4-dinitrophenylhydrazine derivatives such as bis-hydrazones which form with compounds such as sugar osones, glyoxal, diacetyl and methyl glyoxal.[13] It is not to be assumed that only bis-hydrazones give blue colors in alkaline ethanol solutions, but the fact that the blue color change is associated with bis-hydrazones and osazones is of special interest in view of the fact that apricots contain high concentrations of sugars. The possibility that sugar derivatives may be among these unknowns should not be overlooked.

Acknowledgment.—The author wishes to express his appreciation for the kind assistance and advice of Dr. G. Mackinney.

Summary

1. A method has been described for the separation and identification of 2,4-dinitrophenylhydrazones using spectroscopic and chromatographic methods.

2. Furfural and hydroxymethylfurfural have been shown to be present among the intermediate compounds formed during the browning of apricots.

3. In addition to furfurals at least 13 other carbonyl compounds are removed from apricot sirup by continuous extraction with ethyl acetate. To what extent carbonyl compounds other than the furfurals are involved in browning reactions remains to be demonstrated.

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(13) Neuberg and Strauss, *Arch. Biochem.*, **11**, 457 (1946); **7**, 211 (1945).

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The Deterioration of Dried Fruit. IV. Spectrophotometric and Polarographic Studies

BY G. MACKINNEY AND ODETTE TEMMER

It has been shown that the so-called "browning reaction" in many dried fruits is non-enzymatic, and the effect of certain variables (oxygen, sulfur dioxide, moisture and temperature) has been reported.[1] Aqueous extracts of darkened dried apricots, like many products, frequently show a prominent absorption maximum, *ca.* 285 mμ. This band is due to carbonyl compounds not originally present, and sugars contribute significantly to the formation of compounds with this absorption characteristic.[2] Cantor and Peniston[3] have identified hydroxymethylfurfural in deteriorating sirups, while the mechanism of its formation from hexose and the catalytic role of amino acids has been studied by Wolfrom and Cavalieri.[4] Chromatographic procedures applied to the dinitrophenylhydrazine derivatives[5] isolated from apricot extracts indicate a multiplicity of components, of which as yet only furfurals have been identified. It has become necessary to examine the absorption characteristics of several aldehydes and ketones which may arise from degradation of sugar, uronic or ascorbic acids, and which may be present in the deteriorated fruit. The primary purpose of the survey has been to consider under uniform conditions, the extent to which the various compounds tested can contribute to the absorption observed, *ca.* 285 mμ, and the conditions under which they can be detected.

A voluminous literature exists on the absorption spectra of carbonyl compounds[6] but the data, obtained on different instruments, are scattered, various solvents were used, discrepancies are to be found, and important points may be overlooked. Thus Lewis and Calvin[7] observed that "fundamental bands of the whole conjugated system may in simpler molecules be of such low intensity as to be frequently overlooked." They added that this was the case with crotonaldehyde, in the work of Hausser, *et al.*[6a] This does not affect the general

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(1) *Ind. Eng. Chem.*, **38**, 99, 324, 541 (1946).

(2) Paper No. I of this series, THIS JOURNAL, **70**, 3576 (1948).

(3) Cantor and Peniston, *ibid.*, **62**, 2113 (1940).

(4) Wolfrom and Cavalieri, *ibid.*, **70**, 514 (1948).

(5) Paper No. III of this series, *ibid.*, **70**, 3583 (1948).

(6) *E. g.* (a) Hausser, Kuhn, Smakula and Hoffer, *Z. physik. Chem.*, **B29**, 371 (1935); (b) Ramart-Lucas, *Bull. soc. chim.*, **51**, 289 (1932); (c) Tables Annuelles Int. de Constantes et Données Numeriques, Paris, 1930, *et seq.*

(7) Lewis and Calvin, *Chem. Revs.*, **25**, 273 (1939).