DETERMINATION OF CARBONYL COMPOUNDS DEVELOPED DURING STORAGE OF FREEZE-DRIED BEEF

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ABSTRACT

DETERMINATION OF CARBONYL COMPOUNDS DEVELOPED DURING STORAGE OF FREEZE-DRIED BEEF

by Toshio H. Saga

This study has been undertaken to investigate whether the determination of carbonyl compounds can measure progressive oxidative changes in stored freeze-dried meats in relation to: degree of change with time, role of monocarbonyl compounds, and the effectiveness of certain antioxidants and their method of application.

For the carbonyl analysis, the Henick method and the Keith and Day method were employed with some modifications. The major modifications involved attempts to extract and measure the carbonyls of the protein bound lipids in freeze-dried meat. Both procedures involved colorimetric determination of carbonyls based on the formation of a colored quinoidal ion of 2,4-dinitrophenylhydrazone derivatives in a basic solution, and calculation by substituting the spectrophotometric data for the quinoidal ion into simultaneous equations. Thus, the major classes of carbonyls were distinguished through calculation by means of the equations from the data for a single solution without actual fractionation into the separate components.

The accumulation of carbonyl compounds in the stored freeze-dried beef was observed to occur to variable degrees. However, the increase of carbonyl compounds could not be definitely correlated to other factors such as the effectiveness of the antioxidants and their method of application.

Due to the complexity of the autoxidation process, it appeared that no simple analytical method could be directly applied to the evaluation of oxidative change in freeze-dried meat with complete satisfaction.

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INTRODUCTION

Rancidity, which can be defined as any off-odor or flavor developed in lipid or fatty portions of food, degrades the quality of food during storage. In this study, this term is confined to oxidative rancidity by atmospheric oxygen.

The history of objective tests for rancidity begins many years ago with development of the Kreis test (35). Although numerous workers have attempted to make quantitative evaluations of rancidity very few of them have proved to be satisfactory. Among the many methods attempted, the carbonyl test appears to have promise for this purpose (12). Carbonyl compounds, especially volatile monocarbonyls, have been directly related to flavor deterioration of oxidizing lipids (5, 19, 33, 43).

In this study the carbonyl content was determined in several samples of freeze-dried raw and cooked beef. Freeze-drying is considered one of the most promising methods for dehydrating and preserving food (7, 22). Freeze-drying, while it protects against microbial spoilage because of the low moisture content, does not prevent the development of oxidative rancidity in the lipid components.

In the first two experimental sections, Henicks' procedure (24) with some modification was employed to

determine carbonyls in freeze-dried meat. The method of Henick differentiates saturated carbonyl from $\alpha\beta$-unsaturated carbonyl by means of substituting absorbancy values at 430 and 460 mμ into equations. In the last two sections, a modification of the method developed by Keith and Day (33) was applied to freeze-dried beef and to a model system with corn oil on freeze-dried egg white. The Keith and Day method makes it possible to distinguish and account for three major classes of free monocarbonyl compounds using simultaneous equations with spectrophotometric data obtained on derivatives of carbonyls. Both procedures (24, 33) involve a colorimetoric method for carbonyl compounds based on the formation of a colored quinoidal ion of 2,4-dinitrophenylhydrazones* of the carbonyl compounds in a solution of base (36).

The aim of this study has been to learn whether the application of the methods for carbonyl compounds can measure the progressive oxidative changes in stored freeze-dried meats in relation to: degree of change with time, role of monocarbonyl compounds, and the effectiveness of certain antioxidants and their method of application.

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*2,4-Dinitrophenyl is hereafter abbreviated DNP.

REVIEW OF LITERATURE

Process of Oxidative Rancidity of Lipids

Among lipid constituents of foods, those containing polyunsaturated fatty acids are most susceptible to autoxidation or spontaneous oxidation by atmospheric oxygen (23, 28). The generally accepted theory for lipid autoxidation is that the oxidation of the unsaturated fatty acid is initiated by the attack of molecular oxygen at a double bond to form a diradical. This diradical activates α-methylene groups of other unsaturated systems, and oxygen attack at these sites leads to the formation of allylic hydroperoxides (3, 16).

It is commonly known that the formation of the hydroperoxides develops through a chain reaction that is considered to occur in three stages, namely initiation, propagation and termination, involving free radicals. The energy necessary to form the very first radical may come from heat, light, or ionizing radiation.

A characteristic feature of lipid oxidation is the ease with which it can be influenced by factors other than the usual ones of concentration of reactant and temperature.

The kind and quantity of unsaturated fatty acid in the lipids affect the rate and course of development of rancidity. Phosphatides which usually contain a greater amount of polyunsaturated fatty acids than the associated neutral lipids are more readily subjected to oxidation (37, 46, 62, 63). An anomaly results from the fact that phosphatides such as lecithin may also act as an antioxidant or synergist (45). This phenomenon may result if the phosphatides react first with any pro-oxygenic substance present and effect the removal of this substance from the oxidizing lipid system (18). Some other so-called synergists such as ascorbic acid may have both functions, that is, they sometimes act as an antioxidant and sometimes as a pro-oxidant, according to various conditions such as pH, concentration of synergist, and so on (41).

Factors which inhibit lipid oxidation are low temperature (59), the presence of antioxidant and synergist (8, 34, 50), the exclusion of oxygen, light, and ionizing radiation (4, 13, 60), low moisture levels (17, 30, 59), and exclusion of trace metals and salts. High temperature (59), exposure to oxygen, light, and ionizing radiation (4, 13, 60), the presence of enzymes and catalysts such as lipoxidase (49), hematin compounds (58), and metals, etc., are all considered to play a role in accelerating the oxidation of lipids.

The hydroperoxides are the primary products of lipid autoxidation. The so-called secondary degradation products

are formed by the dismutation of hydroperoxides (2, 3). Carbonyl compounds are typical secondary products which are usually unstable and may decompose or react further (32).

Badings (3) has published a review of the principles of the autoxidation process in lipids. Appendix III shows monocarbonyls or aldehydes resulting from cleavage of monomeric hydroperoxides formed during autoxidation of different unsaturated fatty acids. Appendix I illustrates the scheme of formation of aldehydes from linoleic acid by a concept first postulated by Bell et al. (3). Aldehydes formed from other unsaturated fatty acids by this scheme are those in Appendix III. These hypothetical components have been proved by many researchers (2, 15, 20, 44, 47) to exist in mildly oxidized esters of unsaturated fatty acids or natural fats and oil. Gaddis et al. (20) have shown that appreciable amounts of heptanal can be isolated from autoxidized lamb and beef fat. They also found heptanal and decanal in oxidizing butterfat, although these could not be theoretically predicted from the four unsaturated fatty acids in Appendix III (40).

Relationship of Rancidity to Carbonyl Value

Although organoleptic evaluation is the ultimate standard of rancidity of foods, it does not lend itself to quantitative measurement or reproducibility. Determination of the degree of rancidity of stored food may indicate the

stability or keeping quality of the food. The most commonly used objective tests to determine the degree of rancidity are: TBA test (7, 9, 10, 54, 64), carbonyl test (5, 24, 33, 36, 42, 48, 53), and peroxide value (1). Among these the carbonyl test appears to be one of the most promising for this purpose, since odors or flavors associated with typical oxidative rancidity are mainly due to carbonyl type compounds (12). The greater part of the carbonyls determinable by the conventional methods (24, 36) are non-volatile carbonyls (5, 19, 38). It has become apparent that many of the carbonylic substances which accumulate as secondary oxidation products are non-volatile and that, like the hydroperoxides, these make little or no major contribution to off-odor or flavor, although they are probably precursors of volatile odorous substances and may well have deleterious effects on stability (5, 12, 19).

McKerrigan (43) reported that volatile carbonyl shows a better correlation with flavor than either the peroxide or total carbonyl value. Volatile carbonyl compounds are usually collected by vacuum or steam distillation (5, 19).

Much attention has recently been directed toward the separation, estimation, and identification of the volatile carbonylic degradation products from oxidizing lipids (21, 33, 44, 53, 55, 61).

Flavor Thresholds of Monocarbonyl Compounds

Flavor thresholds for a series of aliphatic aldehydes have been determined by Lea et al. (39). It was found that the C_8 - C_{12} saturated aldehydes and the C_9 2-enal were detectable in water at dilutions as great as one part in 10^8 or 10^9. This level is similar to the value reported by Patton et al. (47).

Analytical Methods for Carbonyl Content

A widely used analytical method for carbonyl content is the colorimetric DNP-hydrazine procedure of Henick et al. (24). This procedure is a modification of the method introduced by Lappin and Clark (36). The major modifications are the substitution of trichloroacetic acid for hydrochloric acid and the use of benzene as a solvent to make this method universally applicable to fats. The reaction of DNP-hydrazine with a carbonyl compound, and the wine-red chromophoric quinoidal ion developed in the presence of a base are shown in Appendix II (36). Since this color is unstable and the absorbancy decreases with time at a rate of 0.5% to 0.7% per minute, the time element is very important in determinations using this color reaction (31, 48).

Henick et al. (24) determined saturated and α,β-unsaturated aldehydes by absorbancy measurement at 430 and 460 mμ. The values which were obtained were much higher

than those determined by Pool and Klose (48). Data presented by Gaddis et al. (19) indicates that decomposition of carbonyl precursors occurs during the DNP-hydrazine reaction when using Henick's method (24).

Pool and Klose (48) applied a chromatographic mixture of carbonyl compounds to a 15% hydrated alumina column impregnated with DNP-hydrazine, and found that this column retained the excess reagent and the derivatives of polycarbonyl compounds. Monocarbonyl derivatives were eluted and determined colorimetrically.

While the procedures of Pool et al. (48) and Henick et al. (24) possessed certain desirable features, it was observed that neither gave an adequate picture of carbonyl distribution (33). Neither procedure distinguished between the alkanals, alk-2-enals and alk-2,4-dienals which have been found in oxidizing lipids (20, 33).

In 1963, Keith and Day (33) introduced a modification of the Pool and Klose method. Simultaneous equations were derived to distinguish between and account for the three major classes of monocarbonyls (Appendix V). The Keith and Day method (31) also involved formation of DNP-hydrazones on an alumina reaction column and determination of quantity by colorimetry. The values obtained by the procedure have been referred to as free carbonyl (19). It was found that the quantitative composition of the DNP-hydrazone derivatives obtained from milk fat by the alumina reaction column were

identical to the volatile carbonyl fraction (33). Moreover, there was less than 5% difference in the volatile monocarbonyl content of milk fat and the quantity measured by the modified Pool and Klose procedure (33, 48). This procedure did not measure the carbonyls nor those aldehydes present but bound through an enol ether linkage (33, 51).

Stability of Freeze-Dried Meats

Among the dehydration methods applicable to foods, freeze-drying has proved to be highly effective for the maintenance of desirable palatability (7, 22). Some undesirable changes such as decreased tenderness and loss of texture are frequently encountered in foods which have been freeze-dried.

An important application of freeze-drying appears to be for meat products, but, unfortunately, they are among the more difficult foods to freeze-dry successfully (6). Although the primary characteristics of freeze-dried meat, especially for military and emergency use, lies in its storage stability, there are deteriorative changes which occur during extended storage under ordinary conditions. Deterioration of dehydrated foods due to microbial growth is not common because of the low moisture content. Oxidation is involved in most of the undesirable effects which develop during storage.

Tappel et al. (56, 57) have studied deteriorative changes in freeze-dried meats. The most labile components

are lipids, particularly phosphatides with their high content of polyunsaturated fatty acids, and protein bound lipids, which readily undergo the oxidative changes and consequent flavor deterioration (29, 37, 62, 63). Tappel et al. (56) have reported that oxidation of non-ether-soluble conjugated lipids could account for approximately 50% of the total oxygen absorption, and that the most unusual and most deteriorative oxidation reaction in freeze-dried beef appears to involve the oxidation of the protein fraction.

Another reaction limiting the storage life of freeze-dried meat is nonenzymatic browning. Browning reaction of the Maillard type between a carbonyl group and the free amino-groups of protein or amino-acids have long been recognized as potent causes of change in the color and flavor of foods. The several types of browning, the many different reactants and reactions involved in each type, and the large number of controlling variables make the pathway of browning reactions an intricate subject (27).

Lea (37) has suggested that several kinds of phospholipids containing free amino-groups, could undergo the same kind of reactions with sugar and other aldehydes in freeze-dried meat as do the amino-group of amino acids and proteins.

Henrickson et al. (25, 26) have shown that the storage life of dehydrated pork can be extended by reducing the glucose content. Browning has been reported by Tappel et al. (57) to be responsible not only for deteriorative

changes but also for the desirable flavor change of pre-cooked freeze-dried beef during storage.

Flosdorf (17) has reported that moisture content should be reduced to below 2 percent, and preferably to 0.5 percent for best keeping quality while Goldblith et al. (22) have pointed out that storage stability is greater with raw materials of higher quality.

EXPERIMENTAL METHODS AND PROCEDURE

Preparation of Reagents

Carbonyl-Free Benzene. The following two methods were used to purify benzene.

Method I. (For use in Experiments I and II)

Two liters of benzene were refluxed for 1 hour with 10 g. of DNP-hydrazine and 2 g. of trichloroacetic acid, and then distilled through a Vigreux column.

Method II. (For use in Experiments III and IV)

This method is a modification of the method by Schwartz et al. (52). One g. of DNP-hydrazine was dissolved by grinding in a mortar with 12 ml. of 85% H_3PO_4 and 8 ml. of H_2O were added to the clear yellow solution. The precipitated DNP-hydrazine was redissolved by further grinding. Twenty g. of Celite (Johns-Manville, Hyflo Super-Cel) were then ground with the solution until a homogeneous damp preparation was obtained. The bright yellow impregnated Celite was then transferred to a chromatographic tube of I. D. 28 mm. which had a stopcock at the outlet and which contained about 50 ml. of carbonyl-free n-hexane. A faster flow rate was obtained by using Celite 545 instead of Hyflo Super-Cel in some of the preparations. The height of the impregnated Celite

column was 12.5 cm. The carbonyl-free hexane in the column was drained from the column. The benzene to be purified was stirred with excess DNP-hydrazine, and the benzene saturated with DNP-hydrazine was added to the column. After discarding the first 300 ml. of effluent, the remainder of the effluent was collected at a flow rate of 4 liters per 24 hours. The yellow effluent was stirred with adsorptive magnesia (Sea Sorb 43, Fisher Scientific Co., Pittsburgh 19, Pa.) to remove residual DNP-hydrazine, and the mixture was filtered. The filtrate was distilled at atmospheric pressure to obtain carbonyl-free benzene.

Carbonyl-Free Absolute Ethanol. 14 g. of aluminum granules and 18 g. of KOH were added to 2 liters of ethanol. The mixture was refluxed for 1 hour. On distillation, the first 100 ml. of distillate was discarded, and distillation was stopped before the last 100 ml. had distilled.

DNP-Hydrazine Solution. DNP-hydrazine, twice recrystallized from carbonyl-free methanol, was dissolved in amounts of 0.5 g. in 1 liter of carbonyl-free benzene to form a 0.05% solution (W/V).

Trichloroacetic Acid Solution. Forty-three g. trichloracetic acid were dissolved in carbonyl-free benzene and made to 1 liter to form a 4.3% solution (W/V).

Potassium Hydroxide Solution. A 4% solution (W/V) was made by dissolving 4 g. of KOH with stirring in 100 ml. of

absolute carbonyl-free ethanol. This solution was prepared fresh and centrifuged for 10 min. immediately before use at a speed of about 2,000 r.p.m.

Hydrated Alumina. Activated alumina, F-20 grade, (Aluminum Co. of America, East St. Louis, Ill.) was modified by mixing with 15% fully hydrated material prepared by exposing the alumina in a thin layer to water vapor in a vacuum desiccator. The mixed alumina was allowed to stand and equilibrate in a closed container for several hours before use.

Extraction of Carbonyls from Freeze-Dried Meats

A sample of freeze-dried meat weighing 5 - 7 g. was weighed into a homogenizer flask (Virtis '45). Then 20 ml. of carbonyl-free ethanol were added and the mixture was allowed to stand for 1 min. while the ethanol penetrated the meat. Forty ml. of carbonyl-free benzene was then added and the mixture was blended by a Virtis '45 homogenizer at a speed-setting of 50 for 2.5 min. while cooling the flask with water at a temperature of approx. 5°C. The resulting slurry was filtered through a Buchner funnel and the residue in the funnel was washed with approx. 40 ml. of a mixture of benzene and ethanol (2 to 1; v/v). Depending on the concentration of carbonyls, 3 - 5 ml. of the filtrate were pipetted out for analysis of carbonyls.

Determination of Crude Fat Content

Method I. A known volume of a portion of the filtrate which had been used for carbonyl analysis, was transferred into a tared flask with a standard tapered joint. The solvent was removed carefully under vacuum using a Rinco flask evaporator. The flask containing the crude fat was held in a vacuum desiccator overnight, then the gross weight was determined on an analytical balance to calculate the crude fat content.

Method II. The fats, soluble in hydrocarbon solvents, were extracted from dry meat samples using petroleum ether (Boiling range; 30°C - 60°C) in a Soxhlet apparatus.

Determination of Moisture

Approximately 5 g. of ground meat sample were weighed into a tared weighing bottle. The weighing bottle and its contents were kept in a drying oven at a temperature of 100°C at atmospheric pressure for 24 hours. After being cooled in a vacuum desiccator, the weighing bottle was reweighed and the loss in weight was assumed to be due to moisture loss.

Procedure of Cooking and Freeze-Drying

Beef (Commercial grade, Top round) was obtained from the Michigan State University Food Store. It was closely trimmed of depot fat and connecting tissue. The trimmed

meat was sliced into cubes of approximately 2 cm on edge and divided into two lots. One lot was freeze-dried without cooking, and the other was freeze-dried after being cooked in a Radarange Micro-Wave Oven, Model 1161 (Raytheon Manufacturing Co., Newton, Mass.) in a single layer for 5 min.

Both cooked and raw meat samples were freeze-dried in a Stokes Freeze-Dryer, Model 2003-F2 (F. J. Stokes Machine Co., Philadelphia, Pa.). The frozen cubed meat samples were laid on a tray in a single layer, and placed in the freeze-dryer. During the last half period of freeze-drying, the gauge controlling the heating plate was set to control the temperature at approximately 42°C. The vacuum in the drying chamber was observed to fall to a minimum pressure of approximately 0.1 mm. of Hg.

Experiment I.

Determination of Carbonyl Compounds by a Modification of Henick's Method (24)

Preliminary tests were conducted to determine the applicability of the Henick method for carbonyls in freeze-dried meats. The following two meat samples were used. Sample 1: Cooked freeze-dried beef stored in an open glass jar at room temperature (23 ± 2°C.) for approximately 12 months. Sample 2: Cooked freeze-dried beef stored in an open glass jar at room temperature for approximately 5 months. (These were not from the same source.)

The procedure described below details the modified Henick method used (23).

Determination of Two Classes of Carbonyls

Three ml. of trichloroacetic acid solution (4.3%) and 5 ml. of DNP-hydrazine solution (0.05%) were placed in a 50 ml. volumetric flask and then 5 ml. of the benzene-ethanol solution containing the carbonyls to be analyzed was added. The flask was loosely stoppered and heated in a water bath at 60°C. for 30 min., and then cooled to room temperature. To develop the color, 10 ml. of KOH solution was added, carbonyl-free absolute ethanol was added to make a total volume of 50 ml. and all components mixed thoroughly. After exactly 10 min., the absorbancy values of the wine-red colored solution were read at 430 and 460 mμ in a Beckman DU spectrophotometer against a blank prepared in the same manner by substituting 5 ml. of the carbonyl-free benzene-ethanol mixture (2 : 1, v/v).

The absorbancy values at 430 and 460 mμ were substituted into the following equations to calculate the saturated and the α,β-unsaturated carbonyls.

$$\text{Saturated} = 5.803\ A_{430} - 4.412\ A_{460}$$

$$\text{Unsaturated} = -3.366\ A_{430} + 4.338\ A_{460}$$

where A_{430} indicates absorbancy at 430 mμ.

A_{460} indicates absorbancy at 460 mμ.

Unit is μ moles of carbonyl per 50 ml. soln.

Experiment II.

Changes of Carbonyl Content in Freeze-Dried Raw and Cooked Beef during Storage at Room Temperature

Ten different samples of a single lot of freeze-dried beef were prepared, and stored, wrapped with aluminum foil, at room temperature (23 ± 2°C.) for 12 weeks. Controls and antioxidant treated samples were prepared as noted below. During the storage, saturated and α,β-unsaturated carbonyls were determined by a modification of the method by Henick et al. (24).

Sample Preparation for Experiment II.

Sample 1; The cooked meat was dipped into an aqueous solution of 0.01% propyl gallate (P.G.) at approximately 17° C. for 15 min. After being drained on a screen, the P.G.-treated meat was freeze-dried to obtain a final moisture of less than 2% for about 24 hours.

Sample 2; Sample 2 was prepared in the same manner as Sample 1 except that trihydroxybutyrophenone (T.H.B.P.) was used instead of P.G.

Sample 3; The cooked meat was freeze-dried for 8 hours. The partially dehydrated meat was removed from the freeze-dryer, and dipped into an aqueous solution of 0.01% P.G. at approximately 17°C. for 15 min. After being drained, the meat was again placed in the freeze-dryer for an additional 16 hours until the final moisture content was less than 2%.

Sample 4; Procedure was the same as for Sample 3 except that T.H.B.P. was used instead of P.G.

Sample 5; Fifty g. of the freeze-dried cooked beef was placed in a 2.59 liter desiccator together with 7.5 mg. of butylatedhydroxyanisole (B.H.A.). The desiccator was evacuated by a water aspirator for 5 min., and sealed hermetically at approximately 20 mm. Hg at room temperature. In order to make the solid B.H.A. vaporize and penetrate the freeze-dried meat sample, the desiccator was placed in an oven at 60°C. for 20 hours.

Sample 6; Procedure was the same as for Sample 5 except that butylatedhydroxytoluene (B.H.T.) was used instead of B.H.A.

Sample 7; Control sample comparing to the cooked antioxidant-treated samples (Samples 1 - 6).

Sample 8; Freeze-dried raw beef was treated with B.H.A. exactly as the cooked freeze-dried beef in Sample 5.

Sample 9; Freeze-dried raw beef was treated with B.H.T. exactly as the freeze-dried cooked beef in Sample 6.

Sample 10; Control sample to compare with the raw antioxidant-treated samples (Samples 8 - 9).

Experiment III.

Determination of the Classes of Free Monocarbonyl Compounds in Oxidizing Lipids of Freeze-Dried Beef using Benzene-Ethanol Extracts

Different treatments of freeze-dried beef, a model system with corn oil incorporated in freeze-dried egg white, and a pure chemical two component system consisting of hexanal and cinnamaldehyde were used. The preparation of the freeze-dried beef samples was similar to that in Experiment II except for the following two points: (a) In the treatment of B.H.A. or B.H.T. vapor, a vacuum pump was used to evacuate the desiccator for 2 min. The desiccator was sealed hermetically at a pressure of less than 1 mm. Hg at room temperature. (b) Aluminum foil was not used for wrapping the sample. Six g. samples were weighed accurately into 100 ml. beakers. All samples were weighed at the same time, and allowed to stand in the beakers covered with watch glasses at room temperature until analysis.

The following modified procedure of the method by Keith and Day (33) was used in this portion of the study:

Determination of Three Classes of Free Monocarbonyls

A chromatographic tube of 15 mm I.D. x 30 cm. was plugged at the lower constricted end with sintered glass and/or glass wool, and the outlet was clamped. Carbonyl-free benzene was added to a level of 5 cm. and the 15% hydrated

alumina was added to a depth of 3 cm. The tube was tapped lightly to remove air bubbles and benzene was allowed to drain to the level of alumina. Ten g. of DNP-hydrazine reagent were added and sufficient 15% hydrated alumina was immediately added to increase the depth of the column by 1 cm. After the reagent had passed onto the column, an additional 10 ml. of carbonyl-free benzene was added and the total depth of the column was made to 8 cm. by further addition of 15% hydrated alumina. Finally, the column was washed with 5 ml. of carbonyl-free benzene prior to being used. Three to five ml. of the benzene-ethanol solution containing less than 1 μmole of carbonyls was added to the column. The solution was completely washed into the column with small aliquots of carbonyl-free benzene. A total of 100 ml. of carbonyl-free benzene was then percolated through the column at a flow rate of 5 ml. per min. The eluate was collected in a 250 ml. standard taper round bottom flask. The solvent was carefully removed from the flask under reduced pressure, and the following were added in the sequence listed: 5 ml. of carbonyl-free benzene, 10 ml. of 4% ethanolic KOH, 35 ml. of carbonyl-free absolute ethanol. The flask was stoppered and the contents were thoroughly mixed by inverting and shaking. The absorbancy values of the above solution were read at 430, 460, and 480 mμ. commencing exactly 10 min. after addition of the ethanolic KOH. Blank determinations were run at the same time on 100 ml. of the purified benzene and the color pigment in sample. The

blank of color pigment was determined on a portion of the sample which had been passed over an alumina column free of DNP-hydrazine. After accounting for the absorbancy of the benzene blank and the pigment of the sample, the quantity of each of three monocarbonyl classes was calculated by means of the following simultaneous equations: (See Appendix V for derivation of constants).

$$\text{Alkanals} = 7.158\ A_{430} - 11.142\ A_{460} + 6.496\ A_{480}$$

$$\text{Alk-2-enals} = -5.477\ A_{430} + 15.374\ A_{460} - 11.090\ A_{480}$$

$$\text{Alk-2,4-dienals} = 1.514\ A_{430} - 6.634\ A_{460} + 6.423\ A_{480}$$

where A_{430} indicates absorbancy at 430 mμ., etc.
Unit is μmoles of carbonyl per 50 ml. solution.

Preparation of a Model System with Corn Oil Incorporated in Freeze-Dried Egg White

Raw egg white was beaten, and corn oil was added to the whipped egg white to a level of 5% of raw egg white by weight. The mixture was whipped until firm, and then held at -40°C. for 1 hour. The frozen sample was transferred to the freeze-dryer, where it was dried for 11 hours to obtain a final moisture content of 2% with the heating plate temperature of approximately 45°C. under a vacuum of approximately 0.1 mm. Hg.

The finished sample was transferred into a brown glass bottle with a large head space and stored at room temperature (23 ± 2°C.) until analysis.

Experiment IV.

Determination of the Classes of Free Monocarbonyl Compounds in Oxidizing Lipids of Freeze-Dried Beef using an Acid-Benzene Mixture as Extracting Means

The freeze-dried beef samples were prepared in the same way as in Experiment III. In extraction of carbonyls, pure benzene and an acid-benzene mixture were used. The method using the pure benzene was essentially that of Keith and Day (33). The method using the acid-benzene mixture was as follows:

Extraction of Carbonyls with an Acid-Benzene Mixture

Seven g. of sample were placed in a 100 ml. homogenizer flask (Virtis '45) and then 30 ml. of 15% H_2SO_4 (5.64 N) and 60 ml. of carbonyl-free benzene were added. The mixture was homogenized with a dial setting of 45 on the Virtis '45 homogenizer for 2.5 min. The slurry was transferred to a centrifuge bottle with the aid of a small amount of carbonyl-free benzene, and centrifuged at a speed of 2,000 r.p.m. for 10 min. The supernatant was transferred to a 250 ml. graduated cylinder. Forty ml. of carbonyl-free benzene were added to the centrifuge bottle containing the lower phase, this was thoroughly mixed with a spatula. The mixture was again centrifuged in the same manner. The supernatant was combined with the previously obtained solution, and the total volume of the extract was measured.

A 3 - 5 ml. aliquot containing less than 1 μmole of carbonyls was used for analysis following the procedure described in the previous section. For a control sample, the 15% H_2SO_4 was replaced by distilled water, and the remaining procedure was exactly as previously described.

RESULTS AND DISCUSSION

The experiments in this study were conducted on the premise that since carbonyl compounds have been correlated with rancidity in some systems, the degree of rancidity of freeze-dried beef could be evaluated by determining its carbonyl content, especially the monocarbonyls (5, 19, 33, 43). In assessing the results of this investigation, a number of general observations have been made as well as those pertinent to each experiment.

The change in moisture level in the freeze-dried beef probably has some effect on carbonyl formation. When the freeze-dried beef was exposed to the atmosphere during storage, the level of moisture fluctuated and increased from 1.15% to as much as 10.55% according to the humidity of the air. The procedure of weighing all samples at one time, which was mentioned in Experiment III, eliminated the weight errors due to moisture change. In freeze-drying the meat samples, low moisture levels were obtained with less difficulty in the precooked sample than in the uncooked.

In measuring the absorbancy values of quinoidal ion, it was necessary, in order to obtain dependable and reproducible results, to make all absorbancy readings at the same time interval after adding ethanolic KOH. This was

required because the unstable colored quinoidal ion faded with time at a rate of 0.5% to 0.7% per min. (31, 48). A 60 second period was maintained between the readings of the absorbancy values for each sample.

In Experiment III, it was observed that an appreciable amount of carbonyl compounds was released from a rubber tube attached to the lower end of the chromatographic column. A rubber tube, 5 mm. I. D. x 5 cm., liberated an average of 0.145 μmoles of carbonyls by passing 100 ml. of benzene through the tubing at a flow rate of 5 ml. per min. Therefore, in Experiment IV a Teflon stopcock replaced the clamped rubber tube thus eliminating the source of extraneous carbonyl compounds.

In the simultaneous equations (Appendix V) used in Experiments III & IV, the coefficients for the absorbancy values were slightly smaller, by about 0.1%, than those reported in the paper by Keith and Day (33). This small difference did not appear to be grossly meaningful in evaluating results but it seemed to show that the constants used by Keith and Day were not precisely determined.

Initial studies were made using a modification of the method of Henick *et al.* (24). These began with evaluation of known substances. An adequate recovery of carbonyl at a range of less than 250 x 10^{-6} molar concentration of carbonyls in a one-component system using this method is shown in Table 1. When applied to freeze-dried beef, as shown by the data in Table 2, no definite correlation was

made between the period of storage and the amount of carbonyls determined in two different samples of freeze-dried beef. This could have been due to a number of factors which include difference in composition, difference in storage conditions over the period of storage, and difference in condition of the meat prior to preparation and freeze-drying.

Table 1. Standardization of Henick method with hexanal.

Molarity of Hexanal		Percentage of Recovery
Added	Found	
41×10^{-6}	44×10^{-6}	107.2
51×10^{-6}	48×10^{-6}	94.1
82×10^{-6}	86×10^{-6}	104.9
102×10^{-6}	110×10^{-6}	107.8
204×10^{-6}	228×10^{-6}	111.8

Table 2. Carbonyls of freeze-dried cooked beef by the modified Henick method.

Samples	Storage	Saturated* Carbonyls	Unsaturated* Carbonyls	Total* Carbonyls
1	12 months	50.2	69.6	119.8
2	5 months	60.4	85.0	145.4

*Unit: μmoles of carbonyls in 1 g. of sample on dry basis.

The data in Table 3 obtained by the modified Henick method demonstrates the short term erratic changes of

Table 3. Changes of carbonyl content in freeze-dried beef during storage in aluminum foil at room temperature (23 ± 2° C.).

Samples**	Carbonyl* Compounds	Periods of Storage				
		0 week	1 week	2 weeks	4 weeks	12 weeks
1	Saturated	4.56	5.35	7.00	3.08	4.64
	Unsaturated	0.63	1.16	1.73	0.27	0.93
	Total	5.19	6.51	8.73	3.35	5.57
2	Saturated	5.20	4.72	5.45	6.12	6.75
	Unsaturated	0.17	0.67	0.99	-1.35	0.68
	Total	5.37	5.39	6.44	4.77	7.43
3	Saturated	4.46	5.42	3.48	3.57	5.95
	Unsaturated	0.73	0.95	1.28	0.00	1.96
	Total	5.19	6.37	4.76	3.57	7.91
4	Saturated	5.08	5.34	6.05	4.58	7.80
	Unsaturated	0.32	0.83	0.72	0.18	0.12
	Total	5.40	6.17	6.77	4.76	7.92
5	Saturated	1.03	2.89	5.35	5.55	7.80
	Unsaturated	2.42	0.44	0.53	0.27	0.67
	Total	3.45	3.33	5.88	5.82	8.47
6	Saturated	3.44	4.44	5.18	5.64	7.84
	Unsaturated	-0.11	-0.04	0.37	0.16	0.36
	Total	3.33	4.40	5.55	5.80	8.20
7	Saturated	6.79	6.32	5.47	3.11	5.96
	Unsaturated	0.06	0.96	1.79	0.15	1.04
	Total	6.85	7.28	7.26	3.26	7.00
8	Saturated	2.04	4.45	4.74	5.50	7.97
	Unsaturated	1.50	-0.22	0.51	0.22	1.23
	Total	3.54	4.23	5.25	5.72	9.20
9	Saturated	3.90	5.34	4.62	5.49	5.71
	Unsaturated	-0.01	-0.18	0.26	0.19	0.32
	Total	3.89	5.16	4.88	5.68	6.03
10	Saturated	4.05	5.27	5.13	5.01	7.22
	Unsaturated	-0.23	0.02	0.21	0.10	0.31
	Total	3.82	5.29	5.34	5.11	7.53

*Unit is μmoles of carbonyls in 1 g. of meat sample on dry basis.

**Sample numbers refer to the sample preparations described in the section on Experiment II.

carbonyl content in freeze-dried beef both raw and cooked. Antioxidant treated and untreated samples appeared to behave similarly. The effect of the antioxidants in suppressing oxidation in freeze-dried beef could not be detected by this analytical method. When analysis of variance (11) was applied to the experimental data for the total carbonyls in Table 3, there was no significant difference between any two of the different treatments even at the 5% level as judged by the total carbonyl content. Values of the carbonyl content in all samples went up by the end of 12 week storage. These are denoted by a larger calculated F-value 8.15 than the tabular value 3.89 at 1% level (Degrees of freedom: 4 for numerator; 36 for denominator).

In Table 2, the crude fat content by Method I in Samples 1 and 2, were 30.8% and 20.3%, on a dry basis, respectively. The crude fat content of the fresh beef sample used in Experiment II was 17.5% on a dry basis. The lower fat content of the beef used in Experiment II appeared to be manifested by the lower levels of carbonyl content in the data for freeze-dried product as shown in Table 3 than for those shown in Table 2. The average value of the crude fat content in the raw beef was 15.35% for that used in Experiment III, and 18.8% for that used in Experiment IV. It appeared that the level of carbonyl content in a given sample was influenced to some degree by the crude fat content of the sample.

The formation of DNP-hydrazone derivatives in the benzene-ethanol solution containing trichloroacetic acid and the DNP-hydrazine reagent was carried out by refluxing at a temperature of 60°C. During the reaction, decomposition of carbonyl precursors probably occured, and the carbonyl values obtained by this method were much higher than those determined by using the alumina reaction column. This decomposition of precursors was probably partially responsible for the higher levels of the total carbonyls found in Experiment II than those in Experiment III.

While the procedure of Henick et al. (24) possessed the desirable features of simplicity and convenience, this procedure was found not to give an adequate picture of carbonyl distribution in the oxidizing freeze-dried beef. Since it was reported that alkanals, alk-2-enals, and alk-2,4-dienals were the major classes of monocarbonyls in oxidizing lipids by several researchers (15, 20, 33), a modification of the method of Keith and Day, which afforded the separation of these classes of carbonyls, was employed in Experiments III and IV.

After investigation with several kinds of samples in the portion of the study devoted to Experiment III, it was found that the solvent system used to liberate the protein bound lipids had an unfortunate eluting effect on the alumina reaction column impregnated with the DNP-hydrazine reagent. The ethanol washed down undesirable substances such as the unreacted DNP-hydrazine reagent and the DNP-hydrazone

derivatives of polycarbonyls from the column. The elution of the unreacted DNP-hydrazine reagent was recognized by the fast migration of a yellow band in the column, and by the higher absorbancy values compared to those of a control in which the pure benzene used. The elution of the DNP-hydrazone derivatives of polycarbonyls from the column was also demonstrated by the higher absorbancy values.

The model system in Experiment III was set up to examine whether it might be possible to eliminate some of the unknown factors which influence the determination of carbonyls. Due to the unfortunate eluting effect of the solvent system used, the results obtained by the model system with corn oil incorporated in freeze-dried egg white were difficult to correlate with other observations.

The simultaneous equations used in this study (Appendix V) were valid only for the three component system consisting of alkanals, alk-2-enals, and alk-2,4-dienals formed under the same conditions as those for which the equations had been developed. The eluate obtained by the use of the benzene-ethanol solvent system was found to contain more than three components. It was also found that the simultaneous equations did not distinguish between the three major classes of monocarbonyls with significant accuracy. However, the total monocarbonyl content still showed a steady increasing tendency.

Typical results obtained in Experiment III are shown by the data for freeze-dried raw beef in Table 4. These

showed a maximum in carbonyl content after a 9 day storage period. Although many carbonyl compounds were formed and measured, the monocarbonyl content still appeared to be low level, and most of the monocarbonyls measured were the alkanals or saturated aldehydes. The non-detectable values in Table 4 indicated that the difference in absorbancy values of the blank and the sample was not significant. In order for a maximum value to be developed and followed by lower values, the carbonyl formed must have decreased either through volatilization, which is improbable for the higher molecular weight entities, or they must have been reacted with other components of the system, or they were possibly oxidized further to products which were non-carbonyl in nature.

Table 4. Changes of free monocarbonyls in oxidizing freeze-dried raw beef stored at room temperature (23 ± 2°C.)

Storage	Alkanals*	Alk-2-enals*	Alk-2,4-dienals*	Total Carbonyls*
0 day	---**	---**	---**	---**
1 day	---**	---**	---**	---**
2 days	0.012	-0.067	-0.021	-0.076
7 days	0.174	0.310	-0.114	0.370
9 days	1.570	-0.320	0.160	1.410
13 days	0.700	-0.044	0.205	0.861

*Unit is μmoles in 1 g. of sample meat on dry basis.

**Dash marks refer to non-detectable values.

After discovering the eluting effect of ethanol on the products developed on the alumina reaction column, the original Keith and Day method (33) using only benzene as extracting solvent, was again applied to a series of freeze-dried beef samples, but the free monocarbonyl contents remained practically nil for a 20 day storage period at room temperature (23 ± 2°C.) in beakers. This negative result did not indicate that the free monocarbonyl compounds were not formed in the oxidizing samples, but probably meant that the free monocarbonyls extractable by neutral solvent did not accumulate as stable compounds. It could indicate also that many of the monocarbonyl compounds measured in other experiments were not present as such in the samples but were liberated by the conditions of extraction and derivative formation from oxidized precursors.

The lipid fractions primarily involved in the oxidative reaction are phospholipids and protein bound lipids, rather than triglycerides which are generally termed neutral lipids (63). It is often found that the phospholipids from a particular tissue are appreciably more unsaturated than triglycerides from the same source (29, 37, 62). Protein bound lipids which are referred to as proteolipids cannot be extracted readily with the usual fat solvents. Phospholipids which have higher polarity than neutral lipids, and which are more likely to be bound to protein, are practically insoluble in a nonpolar solvent such as benzene. Protein bound lipids appear to share a role with proteins in

maintaining the integrity of the cell. Bonding by water molecules seems to play an important part in the union between the lipid and the protein. Therefore, polar solvents such as methanol or ethanol, which are dehydrating agents, are required for the liberation and extraction of tissue lipids, and even with the use of a polar solvent some of the tenaciously bound lipids appear not to be completely liberated (37, 46, 63).

An acid-benzene mixture was employed as liberating and extracting solvent system in Experiment IV. As shown in Table 5, the carbonyls from the acid-benzene extraction were higher than those from the water benzene extraction, in which the freeze-dried, uncooked beef stored at a temperature of ca. $-12^{\circ}C$. for a month was used as sample. The extraction by the acid-benzene mixture yielded an extra 21% crude fat compared to that by the water-benzene mixture. The color of the extract obtained by the acid-benzene mixture was more intense pinkish-yellow than that by the benzene from the water slurry. The acid treatment thus appeared to liberate the protein bound lipids. However, 15% acid probably caused enough deterioration of oxidized precursors in the meat samples to form additional carbonyls. In order to obtain a more precise evaluation of this acid-benzene extraction method, further study is needed.

Table 5. Monocarbonyls in crude fats extracted from freeze-dried raw beef by an acid-benzene mixture and by benzene from a water slurry.

Solvents	Alkanals*	Alk-2-enals*	Alk-2,4-dienals*	Total Carbonyls*
Acid-Benzene	0.434	-0.003	0.018	0.449
Water-Benzene	0.083	-0.025	0.007	0.065

*Unit is μmoles in 1 g. of meat sample on dry basis.

The accumulation of carbonyl compounds in the stored freeze-dried beef has been observed to a certain extent as shown in Tables 3 and 4. The increase of the carbonyl compounds with storage time, as yet, can not be correlated to other factors such as the effectiveness of the antioxidants and their methods of application. Due to the complexity of the autoxidation process, it seems that no simple analytical method can be directly applied to the evaluation of change in the freeze-dried meat with complete satisfaction.

As an example of what could be considered in a further study, the procedure used in Experiment III could be modified as follows (14): Extract the freeze-dried meat sample with a benzene-ethanol mixture. Let the extract containing essentially total lipids and all kinds of carbonyls react in the alumina column impregnated with DNP-hydrazine reagent by percolating the benzene-ethanol extract through the column. Remove the solvent from the eluate containing

all kinds of DNP-hydrazone derivatives and the unreacted DNP-hydrazine reagent. Dissolve the dry material in carbonyl free benzene and percolate the benzene solution over an alumina column without any DNP-hydrazine reagent in order to separate the DNP-hydrazone derivatives of monocarbonyls from those of polycarbonyls and the unreacted DNP-hydrazine reagent. The eluate containing the DNP-hydrazone derivatives of monocarbonyls would be used to measure absorbancy at 430, 460 and 480 mμ. The three absorbancy values would be substituted into the simultaneous equations with appropriate constants to calculate the three major classes of monocarbonyls.

This modified procedure still includes the treatment with a dehydrating agent such as ethanol which ruptures the lipid-protein linkage. It suggests a means whereby the absorbancy due to polycarbonyls and the unreacted DNP-hydrazine reagent could be eliminated and permit measuring the three classes of monocarbonyls.

CONCLUSIONS

1. The carbonyl values of stored freeze-dried beef as determined by the method of Henick *et al.* (24) were much higher than those obtained by that of Keith and Day (33). While the former method determined the monocarbonyls as well as the polycarbonyls, the latter measured only the monocarbonyls. However, the different values obtained by use of the two methods indicated that decomposition of carbonyl precursors probably occured with the Henick method giving a higher yield of measurable carbonyl.
2. Although many carbonyls were found and measured in the oxidizing lipids in freeze-dried beef, only a few percent of the total carbonyls appeared to be the free monocarbonyls. Most of the free monocarbonyl compounds seemed to be the alkanals or the saturated aldehydes.
3. Carbonyl compounds were observed to accumulate in the stored freeze-dried beef. This increase with time could not be correlated with other factors such as the effectiveness of the antioxidants used in this study and their methods of application.
4. The effectiveness of the antioxidants applied to the freeze-dried beef could not be detected from the analyses of carbonyl content by the methods used in this study.

An analysis of variance denoted no significant difference even at the 5% level between any two of the ten samples treated in the various ways.

5. The two methods employed in this study, namely the modified Henick method and the modified method of Keith and Day, possessed certain desirable features. The main feature were the simplicity and convenience by which major classes of carbonyls were distinguished through calculation by simultaneous equations from data for a single sample without actual fractionation into the separate components.

6. The presence of ethanol cannot be tolerated in the solvent used for eluting DNP-hydrazones from a column since it has the undesirable feature of eluting more than the monocarbonyl derivatives from the alumina reaction column impregnated with the DNP-hydrazine reagent. The ethanol eluted the DNP-hydrazone derivatives of polycarbonyls and the unreacted DNP-hydrazine reagent in addition to the DNP-hydrazone derivatives of monocarbonyls. The absorption data obtained from the solution containing more than the three major classes of monocarbonyls, that is, alkanals, alk-2-enals, and alk-2,4-dienals, could not be applied to the simultaneous equations developed under the three component system in order to calculate properly the three major classes with significant accuracy.

7. Further research was indicated for studies of the acid-benzene extraction method on the long storage series, and of the further modified Keith and Day method which included extraction by a mixture of ethanol-benzene.

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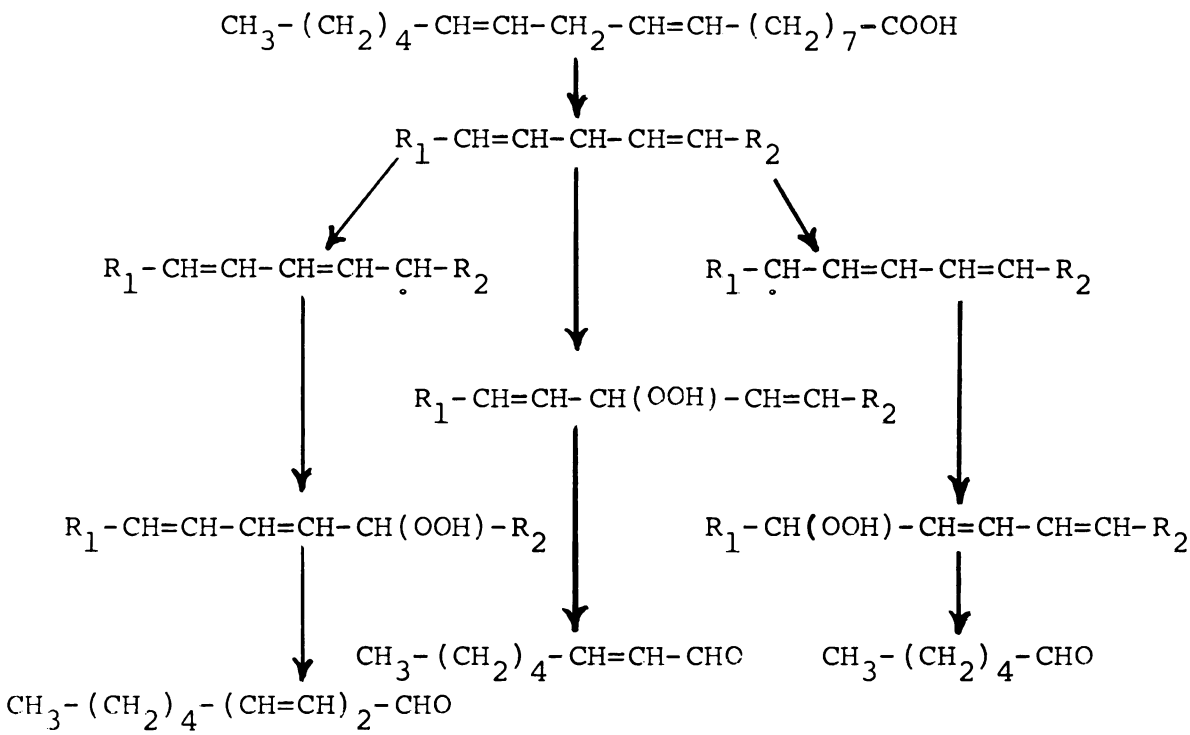
APPENDICES

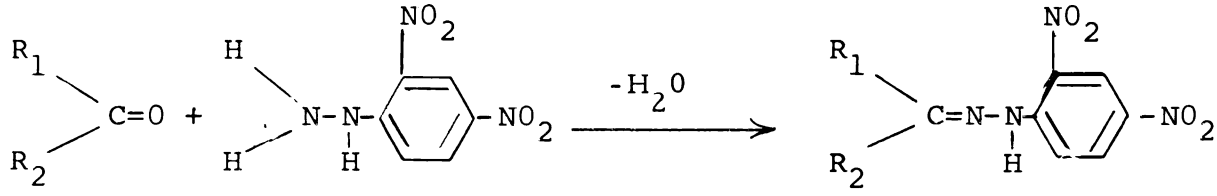
APPENDIX I

Scheme for Formation of Hydroperoxides and Dismutation to Monocarbonyl Compounds

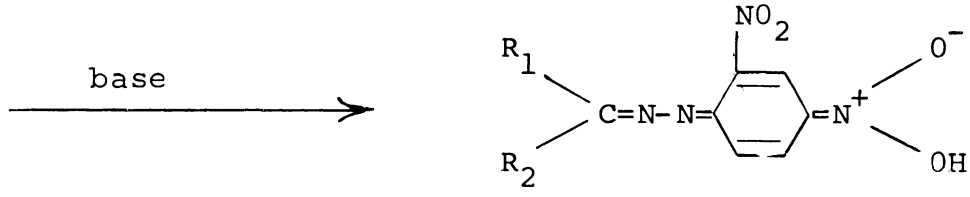


APPENDIX II

Reaction of DNP-Hydrazine with a Carbonyl Compound and the Chromophoric Quinoidal Ion Developed in the Presence of Base



carbonyl DNP-hydrazine DNP-hydrazone derivative



chromophoric quinoidal ion of DNP-hydrazone derivative

APPENDIX III

Hydroperoxides and Aldehydes Derived from Fatty Acids
by the Scheme in Appendix I

Fatty Acids	Hydroperoxides	Aldehydes
Oleic Acid C_{18}-$\triangle^{9}$-	11-hydroperoxy $\triangle^{9}$- 10-hydroperoxy $\triangle^{8}$- 9-hydroperoxy $\triangle^{10}$- 8-hydroperoxy $\triangle^{9}$-	C_8-al C_9-al C_{10}-$\triangle^{2}$-enal C_{11}-$\triangle^{2}$-enal
Linoleic Acid C_{18}-$\triangle^{9,12}$-	13-hydroperoxy $\triangle^{9,11}$- 11-hydroperoxy $\triangle^{9,12}$- 9-hydroperoxy $\triangle^{10,12}$-	C_6-al C_8-$\triangle^{2}$-enal C_{10}-$\triangle^{2,4}$-dienal
Linolenic Acid	16-hydroperoxy $\triangle^{9,12,14}$- 14-hydroperoxy $\triangle^{9,12,15}$-	C_3-al C_5-$\triangle^{2}$-enal

C_{18}-$\triangle^{9,12,15}$-	13-hydroperoxy$\triangle^{9,11,15}$- 12-hydroperoxy$\triangle^{9,13,15}$- 11-hydroperoxy$\triangle^{9,12,15}$- 9-hydroperoxy$\triangle^{10,12,15}$-	C_6-$\angle^{3}$-enal C_7-$\angle^{2,4}$-dienal C_8-$\angle^{2,5}$-dienal C_{10}-$\triangle^{2,4,7}$-trienal
Arachidonic Acid C_{20}-$\triangle^{5,8,11,14}$-	15-hydroperoxy$\triangle^{5,8,11,13}$- 13-hydroperoxy$\triangle^{5,8,11,14}$- 12-hydroperoxy$\angle^{5,8,10,14}$- 11-hydroperoxy$\angle^{5,8,12,14}$- 10-hydroperoxy$\angle^{5,8,11,14}$- 9-hydroperoxy$\triangle^{5,7,11,14}$- 8-hydroperoxy$\triangle^{5,9,11,14}$- 7-hydroperoxy$\triangle^{5,8,11,14}$- 5-hydroperoxy$\triangle^{6,8,11,14}$-	C_6-al C_8-$\triangle^{2}$-enal C_9-$\triangle^{3}$-enal C_{10}-$\triangle^{2,4}$-dienal C_{11}-$\triangle^{2,5}$-dienal C_{12}-$\triangle^{3,6}$-dienal C_{13}-$\triangle^{2,4,7}$-trienal C_{14}-$\triangle^{2,5,8}$-trienal C_{16}-$\triangle^{2,4,7,10}$-tetraenal

APPENDIX IV

Common and Chemical Designations of Antioxidants

Name and Abbreviation	Chemical Names
Propyl gallate P.G.	3,4,5-trihydroxypropylbenzoate
Trihydroxybutyrophenone T. H. B. H.	2,4,5-trihydroxybutyrophenone
Butylatedhydroxyanisole B. H. A.	a mixture of the isomers 2-tert-butyl-4-methoxyphenol 3-tert-butyl-4-methoxyphenol
Butylatedhydroxytoluene B. H. T.	2,6-di-tert-butyl-p-cresol

APPENDIX V

Derivation of Simultaneous Equations by Matrix Inversion from Average Molar Absorptivities of DNP-Hydrazone Derivatives of Monocarbonyl Compounds

The average molar absorptivities of alkanals, alk-2-enals, and alk-2,4-dienals for the three wave lengths corresponding closely to the absorption maxima of the DNP-hydrazone derivatives, are given as follows:

	430 mμ.	460 mμ.	480 mμ.
Alkanals	= 20,930	15,290	10,860
Alk-2-enals	= 23,670	30,050	25,460
Alk-2,4-dienals	= 19,700	36,420	40,760

The Lambert-Beer law is mathematically expressed in the form:

$$A = aC_1 + bC_2 + \ldots$$

where **A** is the absorbancy ($\log\frac{100}{\%T}$) of the sample at the given wave length, a, b, etc. are the absorbancys for each of the components in unit concentration, and C_1, C_2, etc. are the concentration for each component.

Hence, in a three component system with the three wave lengths, that is, 430, 460 and 480 mμ;

$$\left.\begin{aligned} A_{430} &= a_{430}C_1 + b_{430}C_2 + d_{430}C_3 \\ A_{460} &= a_{460}C_1 + b_{460}C_2 + d_{460}C_3 \\ A_{480} &= a_{480}C_1 + b_{480}C_2 + d_{480}C_3 \end{aligned}\right\} \quad \ldots\ldots\ldots\ldots\ldots\ldots(1)$$

where A_i's are the absorbancys of the sample consisting of three components at the wave length of i $m\mu$, a_i's are the average molar absorptivities of alkanals at the wave length of i $m\mu$, and similarly b_i's and d_i's are the average molar absorptivities of alk-2-enals and alk-2,4-dienals, respectively.

The given average molar adsorptivities are substituted into equations (1).

$$\left.\begin{aligned} A_{430} &= 20{,}930\ C_1 + 23{,}670\ C_2 + 19{,}700\ C_3 \\ A_{460} &= 15{,}290\ C_1 + 30{,}050\ C_2 + 36{,}420\ C_3 \\ A_{480} &= 10{,}860\ C_1 + 25{,}460\ C_2 + 40{,}760\ C_3 \end{aligned}\right\} \quad \ldots\ldots\ldots(1')$$

The above simultaneous equations are inverted by Cramer's rule.

$$C_1 = \frac{\begin{vmatrix} A_{430} & 23{,}670 & 19{,}700 \\ A_{460} & 30{,}050 & 36{,}420 \\ A_{480} & 25{,}460 & 40{,}760 \end{vmatrix}}{\begin{vmatrix} 20{,}930 & 23{,}670 & 19{,}700 \\ 15{,}290 & 30{,}050 & 36{,}420 \\ 10{,}860 & 25{,}460 & 40{,}760 \end{vmatrix}} = \frac{\begin{vmatrix} A_{430} & 23{,}670 & 19{,}700 \\ A_{460} & 30{,}050 & 36{,}420 \\ A_{480} & 25{,}460 & 40{,}760 \end{vmatrix}}{2{,}078{,}735{,}680{,}000}$$

$$= \frac{2{,}975{,}848\ A_{430} - 4{,}632{,}272\ A_{460} + 2{,}700{,}764\ A_{480}}{20{,}787{,}356{,}800}$$

Unit is converted from molar concentration to μmoles per 50 ml. by multiplying by 5×10^4.

$$50{,}000C_1 = 7.158\ A_{430} - 11.142\ A_{460} + 6.496\ A_{480} \quad \ldots\ldots\ldots(2)$$

Similarly, for C_2 and C_3,

$$50{,}000C_2 = \frac{\begin{vmatrix} 20{,}930 & A_{430} & 19{,}700 \\ 15{,}290 & A_{460} & 36{,}420 \\ 10{,}860 & A_{480} & 40{,}760 \end{vmatrix}}{2{,}078{,}735{,}680{,}000} \times 50{,}000$$

$$= -5.477\ A_{430} + 15.374\ A_{460} - 11.090\ A_{480} \quad \ldots\ldots(3)$$

$$50{,}000C_3 = \frac{\begin{vmatrix} 20{,}930 & 23{,}670 & A_{430} \\ 15{,}290 & 30{,}050 & A_{460} \\ 10{,}860 & 25{,}460 & A_{480} \end{vmatrix}}{2{,}078{,}735{,}680{,}000} \times 50{,}000$$

$$= 1.514\ A_{430} - 6.634\ A_{460} + 6.423\ A_{480} \quad \ldots\ldots\ldots(4)$$

Therefore, from (2), (3) and (4) the simultaneous equations, which give the three major classes of monocarbonyls in terms of μmoles per 50 ml., are as follows:

$$\begin{cases} \text{Alkanals} = 7.158\ A_{430} - 11.142\ A_{460} + 6.496\ A_{480} \\ \text{Alk-2-enals} = -5.477\ A_{430} + 15.374\ A_{460} - 11.090\ A_{480} \\ \text{Alk-2,4-dienals} = 1.514\ A_{430} - 6.634\ A_{460} + 6.423\ A_{480} \end{cases}$$