Determination of the Classes of Free Monocarbonyl Compounds in Oxidizing Fats and Oils[1,2]

R. W. KEITH and E. A. DAY, Department of Food Science and Technology, Oregon State University, Corvallis, Oregon

Abstract

Simultaneous equations were derived to distinguish and account for the major free monocarbonyl classes in oxidizing fats and oils. The procedure of Pool and Klose was modified to accommodate the equations. The procedure involves conversion of the free monocarbonyls to DNP-hydrazones by passage of a benzene solution of the sample over an alumina-DNP-hydrazine reaction column. The alkanals, alk-2-enals and alk-2,4-dienals were measured by absorbance readings at 430, 460, and 480 mμ of a benzene alcoholic-KOH hydrazone solution. The average error of the procedure in the analysis of total carbonyls in authentic mixtures was 2.4%.

It was found that the qualitative composition of the DNP-hydrazones obtained from milk fat by the reaction column were identical to the volatile carbonyl fraction. 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. The data suggest that the procedure affords a means of measuring volatile or free monocarbonyls of oxidizing lipids. Since the volatile monocarbonyls are directly related to flavor deterioration of oxidizing lipids, it would appear that the free monocarbonyls, as measured by the modified Pool and Klose procedure, should exhibit a corresponding relationship.

Introduction

PREVIOUS WORK in our laboratory (1) revealed that the concentration of the volatile alk-2-enal class in milk fat gave a high correlation with the intensity of oxidized flavor. Therefore, a procedure for measurement of the various classes that would be adaptable to routine analysis was sought. While the procedures of Pool and Klose (2) and Henick, Benca and Mitchell (3) possessed certain desirable features, it was observed that neither gave an adequate picture of carbonyl distribution. Neither procedure distinguishes the alkanals, alk-2-enals, alk-2,4-dienals and dicarbonyls, which have been found in oxidizing fats and oils (4,5,6,7,8). Both procedures are based upon the original method of Lappin and Clark (9), whereby the 2,4-dinitrophenylhydrazones[3] of the compounds are formed and their absorbance in alcoholic base is measured. Major differences between the two procedures are the conditions for the DNP-hydrazine carbonyl reaction and the methods of expressing carbonyl content. Pool and Klose utilized benzene and 15% hydrated alumina columns charged with DNP-hydrazine. They made no attempt, however, to distinguish between the various classes of carbonyls occurring in the oxidizing lipids. The values obtained by the pro-

cedure have been referred to as free carbonyls (5). Henick et al. carried out the reaction in benzene containing trichloroacetic acid, and DNP-hydrazine. They determined saturated and α,β-unsaturated aldehydes by the absorbance measurements at 430 and 460 mμ. These values are much higher than those determined by the Pool and Klose method. Data from recent work by Gaddis et al. (5) indicates that decomposition of carbonyl precursors occurs during the DNP-hydrazine reaction with the Henick et al. method. Nevertheless, the simplicity of the procedure has given it wide acceptance for the estimation of carbonyl content and/or flavor deterioration of oxidizing lipids.

The purpose of this investigation was to develop a suitable procedure for accurate measurement of the carbonyl classes. This required derivation of equations to distinguish and account for the carbonyl classes, as well as modification of existing methods (2,3), to accommodate the equations.

Experimental and Results

Solvents and Reagents. Carbonyl-free benzene was prepared by the method of Schwartz and Parks (10). Carbonyl-free ethanol and ethanolic KOH were obtained as described by Berry and McKerrigan (11). The DNP-hydrazine solution and 15% hydrated alumina were prepared as described by Pool and Klose, except that the activated alumina was heated to 180C for 48 hr prior to use. Alkanals and alk-2-enals were obtained from commercial suppliers. Alk-2,4-dienals were synthesized by the procedure of Pippen and Nonaka (12). The α-ketoalkanals were prepared by oxidation of the aldehyde analog with selenium dioxide (13). The DNP-hydrazones were purified by column chromatography (14) and authenticated by mixture melting point analysis. The DNP-osazones were purified by column chromatography (15) and verified by melting point and elemental analyses.

Evaluation of the Henick, Benca and Mitchell Procedure. Aliquots of standard benzene solutions of alkanals, alk-2-enals, alk-2,4-dienals and α-dicarbonyls, were analyzed by the Henick et al. procedure. The results shown in Table I demonstrate the inadequacy of the method for measuring the saturated and unsaturated carbonyl content of mixtures.

Attempts were made to improve the accuracy of

[3] 2,4-Dinitrophenyl hereinafter referred to as DNP.

TABLE I

Quantitative Determination of Authentic Carbonyl Mixtures by the Henick Method [a]

Analysis No.	Micromoles of carbonyl added					Micromoles of carbonyl determined		
	Alkanals	Alk-2-enals	Alk-2,4-dienals	α-dicarbonyl	Total	Saturated	Unsaturated	Total
1	0.480	0.420	0.334	0.216	1.450	0.186	0.895	1.081
2	0.480	0.420	0.334	......	1.234	0.263	0.748	0.710
3	0.500	0.428	......	0.440	1.368	0.340	0.557	1.011
4	0.480	......	0.334	0.216	1.030	0.173	0.537	0.897

[a] Henick, Benca and Mitchell (3).

TABLE II

Evaluation of Equations for Simultaneous Determination of DNP-Hydrazones of Three Monocarbonyl Classes

Analysis No.	Micromoles DNP-hydrazones added				Micromoles DNP-hydrazones determined			
	Alk-anals	Alk-2-enals	Alk-2,4-dienals	Total	Alk-anals	Alk-2-enals	Alk-2,4-dienals	Total
1	0.760	0.446	0.302	1.508	0.744	0.423	0.320	1.487
2	......	0.446	0.302	0.748	0.022	0.402	0.321	0.745
3	0.760	......	0.302	1.062	0.763	−0.030	0.320	1.053
4	0.486	0.401	0.274	1.161	0.452	0.451	0.254	1.157
5	0.486	0.401	......	0.887	0.457	0.423	0.005	0.885
6	......	0.401	0.274	0.675	−0.020	0.419	0.273	0.672
7	0.486	......	0.274	0.760	0.492	0.003	0.275	0.770
8	0.652	0.386	0.305	1.343	0.687	0.415	0.265	1.367
9	0.652	0.772	......	1.424	0.672	0.749	0.004	1.425
TOTAL	4.282	3.253	2.033	9.568	4.269	3.255	2.037	9.561

the procedure by derivation of equations that would determine the aforementioned carbonyl classes. Aliquots of benzene solutions, prepared from replicate weighings of DNP-hydrazones of the monocarbonyl classes and DNP-osazones of the α-dicarbonyls, were analyzed individually in the benzene-ethanolic KOH solution of Berry and McKerrigan (11). Average molar absorptivities, calculated for the four wave lengths corresponding closely to the absorption maxima of the DNP-hydrazine derivatives, were as follows:

	$\epsilon\,430$	$\epsilon\,460$	$\epsilon\,480$	$\epsilon\,565$
Alkanals	20,930	15,290	10,860	8,200
Alk-2-enals	23,670	30,050	25,460	11,700
Alk-2,4-dienals	19,700	36,420	40,760	17,300
α-Dicarbonyls	14,320	19,780	27,270	55,130

Using the above data in conjunction with the inversion of a four by four matrix, the following equations were developed.

$$\text{Alkanal} = 7.764\,A_{430} - 11.907\,A_{460} + 7.505\,A_{480} - 1.457\,A_{565}$$

$$\text{Alk-2-enals} = -6.626\,A_{430} + 16.565\,A_{460} - 12.397\,A_{480} + 1.887\,A_{565}$$

$$\text{Alk-2,4-dienals} = 2.185\,A_{430} - 7.482\,A_{460} + 7.540\,A_{480} - 1.613\,A_{565}$$

$$\text{α-Dicarbonyls} = -0.512\,A_{430} + 0.646\,A_{460} - 0.852\,A_{480} + 1.229\,A_{565}$$

The resulting values are in micromoles per 50 ml of solution. The equations were found to accurately predict the quantities of authentic DNP-hydrazine derivatives in mixtures. However, application of the equations to the analyses of mixtures of carbonyls in benzene gave erroneous results. It was subsequently found that elimination of α-dicarbonyls from the carbonyl mixtures afforded accurate measurement of the monocarbonyl classes. The cause of discrepancy in the results was due to the reaction conditions of the Henick procedure which yielded largely the DNP-hydrazones of the α-dicarbonyls at the expense of the DNP-osazones.

The DNP-hydrazones of α-dicarbonyls exhibit ab-

TABLE III

Micromoles of Free Carbonyl Classes Determined by the Modified Pool and Klose Procedure

Analysis No.	Micromoles of carbonyl added				Micromoles of carbonyl determined [a]			
	Alk-anals	Alk-2-enals	Alk-2,4-dienals	Total	Alk-anals	Alk-2-enals	Alk-2,4-dienals	Total
1	0.500	0.428	......	0.928	0.483	0.429	0.011	0.923
2	0.500	......	0.412	0.912	0.502	−0.020	0.396	0.878
3	0.500	0.428	0.412	1.340	0.553	0.403	0.401	1.357
4	......	0.428	0.412	0.840	−0.004	0.455	0.438	0.889
5	......	0.642	0.412	1.054	−0.005	0.655	0.415	1.065
TOTAL	1.500	1.926	1.648	5.074	1.529	1.922	1.661	5.112

[a] Corrected to 100% recovery.

sorption maxima at 500 mμ which results in high values for alkanals and alk-2,4-dienals and low values for alk-2-enals and α-dicarbonyls. A number of reaction conditions including variations in polarity, acidity of the reaction medium, temperature, time, and the reaction column of Schwartz and Parks (10) were evaluated in an attempt to drive the α-dicarbonyl DNP-hydrazine reaction to complete formation of DNP-osazones, but none were successful. In addition, none of the reaction conditions yielded a constant ratio of the two products. It was concluded that the method was inadequate for measuring either carbonyl content or carbonyl distribution in fats and oils, because oxidizing lipids contain relatively large quantities of dicarbonyls and these compounds effect a large error in calculations of carbonyl content.

Modification of the Pool and Klose Procedure. Pool and Klose found that the alumina DNP-hydrazine reaction column retained dicarbonyl derivatives and allowed elution of only the DNP-hydrazones of monocarbonyls. Therefore, we were able to measure simultaneously the three major monocarbonyl classes of oxidizing fats simply by using the column to form the DNP-hydrazones and by derivation of equations to distinguish the DNP-hydrazone classes.

Slight modifications were made in the original method of Pool and Klose and for the purpose of clarity, the entire procedure is given. The chromatographic tube, 12 mm. I. D. × 40 cm., is plugged at the constricted end with glass wool and the outlet is clamped. Benzene is added to a level of 5 cm. and 15% hydrated alumina is added to a depth of 3 cm. The tube is tapped lightly to remove air bubbles and the benzene is allowed to drain to the level of the alumina. Ten milliliters of the DNP-hydrazine reagent is added and sufficient 15% hydrated alumina is immediately added to increase the depth of the column by 1 cm. After the reagent has passed onto the column, an additional 10 ml. of benzene is added and the total depth of the column is made to 8 cm. by addition of 15% hydrated alumina. Finally the column is washed with 5 ml. of benzene prior to being used. Three to five ml. of benzene solution, containing less than 1.0 μmole of carbonyl, is added and completely washed into the column with small aliquots of benzene. A total of 100 ml. of benzene is then percolated over the column and the eluate is collected in a 250 ml. standard tapered Erlenmeyer flask. The solvent is removed from the flask at reduced pressure and the following are added in the sequence listed: 5 ml. of benzene; 10 ml. of 4% ethanolic KOH; 35 ml. of absolute ethanol. The flask is stoppered and the contents thoroughly mixed. The absorbance values are read at 430, 460, and 480 mμ commencing 10 min after addition of the ethanolic KOH. Distilled water is used as the blank. The time element is very important since the chromophores are unstable and absorbance decreases with time (16). The prescribed wave length sequence should always be followed since this was the order used in determining the molar absorptivities in development of the equations. It is necessary to run a blank determination on 100 ml of each new lot of benzene in order to account for traces of carbonyls remaining after purification. Color pigments in many lipids will cause some interference, and it is recommended that absorbance values of a sample aliquot, which has been passed over an alumina column free of DNP-hydrazine, be determined by the prescribed procedure. These values are then deducted from the absorbance readings

of the reacted sample. After accounting for the absorbance of the benzene blank and the pigments of the lipid, the quantity of each of the three monocarbonyl classes may be calculated by means of the following equations.

$$\text{Alkanals}^4 = 7.163\ A_{430} - 11.157\ A_{460} + 6.506\ A_{480}$$

$$\text{Alk-2-enals}^4 = -5.484\ A_{430} + 15,392\ A_{460} - 11.102\ A_{480}$$

$$\text{Alk-2,4-dienals}^4 = 1.516\ A_{430} - 6.641\ A_{460} + 6.428\ A_{480}$$

The equations were developed by a matrix inversion of the previously listed molar absorptivity values for 430, 460, and 480 mμ. Typical results of application of the equations in the analyses of mixtures of pure DNP-hydrazones in benzene-ethanolic KOH solutions are shown in Table II. Although some deviation from the added amounts are apparent in the data, the total quantities added and determined are in good agreement.

The alumina DNP-hydrazine reaction column was evaluated to ascertain the yield of DNP-hydrazones from the three monocarbonyl classes. Benzene solutions of the alkanals, alk-2-enals and alk-2,4-dienals were passed over the column and the DNP-hydrazones in the eluate were measured. Average yields of DNP-hydrazones were: alkanals, 75%; alk-2-enals, 65%; alk-2,4-dienals, 60%. Derivatives of α-dicarbonyls were retained on the column as previously reported (2) and they did not interfere with the analysis of the DNP-hydrazones of monocarbonyls. Table III shows the results of the analysis of carbonyl mixtures by means of the modified procedure. The data were adjusted to 100% yield of DNP-hydrazones by use of the percentage yield values given above. Summation of each carbonyl class for the five analyses indicates that duplicate or triplicate determinations will improve the reproducibility of the procedure.

Evaluation of the Modified Procedure for Measurement of Free Monocarbonyls in Milk Fat. Application of the modified Pool and Klose procedure to the analysis of oxidizing milk fat revealed that the quantities of carbonyls measured by the procedure were comparable to values reported for the volatile fraction by Lillard and Day (1). To clarify this point, two milk fat samples were autoxidized and a quantitative comparison was made of the volatile carbonyls with those isolated by the alumina reaction column. The peroxide values (17) for the two samples, A and B of Table IV, were 30.9 and 5.2, respectively. The volatile monocarbonyls were isolated by the procedure of Day and Lillard (6). The data for the volatile fractions, Table IV, are corrected to 100% distillation of the volatiles and to complete conversion of the carbonyls to DNP-hydrazones. Similarly, free carbonyl values were corrected to 100% conversion of the carbonyls to DNP-hydrazones.

The qualitative composition of the DNP-hydrazones of the volatile and free carbonyl fractions of sample A, Table IV, was studied to ascertain if differences occurred. The DNP-hydrazones of the volatile fraction were separated into classes (18) and members of each class were purified on partition columns (14). The DNP-hydrazones of the free carbonyls, formed by the alumina DNP-hydrazine column, were isolated from the milk fat by the procedure of Schwartz et al. (19). The derivatives were then analyzed in the same manner as the DNP hydrazones of the volatile fraction. The composition of the derivatives of both

TABLE IV
Comparison of Volatile and Free Monocarbonyls in Oxidized Milk Fats

	Alkanals[a]	Alk-2-enals[a]	Allk-2,4-dienals[a]	Totals[a]
Volatile carbonyls:				
Sample A	0.867	0.150	0.055	1.072
Sample B	0.441	0.000	0.000	0.441
Free carbonyls:				
Sample A	0.782	0.161	0.076	1.019
Sample B	0.372	0.030	0.019	0.421

[a] Corrected to 100% recovery and expressed as millimoles per kg fat.

the volatile and free carbonyl fractions were identical to data previously reported (6,8).

Analysis of Free Monocarbonyl Classes in Oxidizing Fats and Oils. Samples of lard, milk fat, corn, cottonseed, and soybean oils were placed in petri dishes and oxidized at 75C. Peroxide values and free monocarbonyl classes were determined at periodic intervals. The results of the analyses are shown in Table V.

Discussion

The results of this investigation illustrate the value of differentiating the various carbonyl classes when attempting to measure these compounds in oxidizing lipids. The Pool and Klose procedure, as modified in this investigation, does not account for all of the carbonyl classes, but it does give reproducible values for what appears to be the major free monocarbonyl classes in oxidizing fats. Accuracy of the derived equations in differentiating the monocarbonyl classes is illustrated in Table II. The data in Table III shows that the equations are applicable for the analysis of monocarbonyl mixtures after conversion to DNP-hydrazones on the alumina reaction column. The average error for analysis of the total free carbonyl classes was less than three per cent.

The values for the total volatile and total free carbonyl fractions of the two milk fat samples, Table IV, agree within 5%. This suggests that the two procedures are measuring the same compounds in the fat, even though greater differences are apparent for individual carbonyl classes in Table IV. Evidence to support this point are: 1. the qualitative composition of the three DNP-hydrazone classes was comparable, by both the alumina DNP-hydrazine reaction column and the vacuum distillation procedures; 2. the aldehydes in fresh milk fat that have been reported by Schogt et al. (20) to be bound to glycerol in an enol-

TABLE V
Free Carbonyls of Oxidizing Fats and Oils

Sample	Storage[a] time	Peroxide number	Carbonyl concentrations[b]			
			Alkanals	Alk-2-enals	Alk-2,4-dienals	Total
Corn oil	0	0.6	0.214	0.040	0.023	0.277
	0	0.9	0.122	0.026	0.023	0.171
	2	26.8	0.450	0.106	0.070	0.626
	7	195	2.930	0.470	0.320	3.720
Cottonseed oil	0	3.3	0.170	0.007	0.025	0.202
	1	22.1	0.177	0.067	0.005	0.249
	2	63.6	0.377	0.077	0.050	0.504
	2	107	0.441	0.134	0.106	0.681
	4	226	1.930	0.370	0.303	2.600
Lard	0	2.5	0.567	0.039	0.023	0.629
	1	20.0	0.785	0.060	0.067	0.912
	2	156	3.800	0.599	0.730	5.130
Soybean oil	1	1.1	0.222	0.013	0.018	0.253
	2	36.2	0.443	0.057	0.081	0.581
	10	433	15.790	4.030	6.530	26.350
Milk fat	0	0	0	0	0	0
	1	1.8	0	0	0	0
	4	39.5	0.911	0.224	0.161	1.300
	5	114	2.770	0.624	0.380	3.770

[a] Days at 75C.
[b] Corrected to 100% recovery and expressed as millimoles/kg of sample.

ether linkage, were not measured by the modified procedure (see Table V). Reaction conditions of the alumina DNP-hydrazine column are not rigorous enough to effect hydrolysis of the bound carbonyls.

Reproducibility of the method is satisfactory and no difficulties were encountered in analyzing various fats and oils, with the exception of marine oils. Attempts to obtain reproducible values on oxidized marine oils failed. The main problem preventing accurate analysis was due to the formation of an interfering red color when obtaining blank readings on oil samples in benzene-ethanolic KOH solutions.

In previous work Lillard and Day (1) found a high correlation between the concentration of volatile alk-2-enals and the oxidized flavor intensity of milk fat. Since the modified Pool and Klose procedure appears to measure the volatile carbonyls, it is believed that values obtained for the alk-2-enals will show a comparable correlation with oxidized flavor intensity of milk fat.

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On the Structure of Highly Unsaturated Fatty Acids of Fish Oils by High Resolution Nuclear Magnetic Resonance Spectral Analysis

TETSUTARO HASHIMOTO, KENKICHI NUKADA,[1] HISAKO SHIINA and TOMOTARO TSUCHIYA, Government Chemical Industrial Research Institute, Tokyo, Hon-machi, Shibuya-ku, Tokyo, Japan

Abstract

Methyl esters of highly unsaturated fatty acid concentrates were prepared from fish oils by the urea-adduct method. The nuclear magnetic resonance spectra of the mixed esters and some related pattern compounds were analyzed. As a result, it was concluded that the structure of highly unsaturated fatty acids has divinylmethane arrangement of the ethylenic bonds and no divinylethane arrangement, and that one methylene group is present between the terminal methyl group and the double bond located at the remotest position from a carboxyl group in the acids.

Introduction

FISH OILS, especially marine fish oils, generally contain great portions of highly unsaturated fatty acids having more than three double bonds. A great number of works have been made regarding the structure of the acids. Many contributions recently worked out suggest that the structure of highly unsaturated fatty acids are classified into two types: The first (structure I) is the structure comprising solely divinylmethane arrangement of the ethylenic bonds

$$(CH=CH-CH_2-CH=CH-).[2]$$
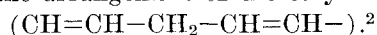
The arrangement is identical to that in linoleic and linolenic acids. The second (structure II) is the structure having solely divinylethane arrangement

$$(-CH=CH-CH_2-CH_2-CH=CH-)$$
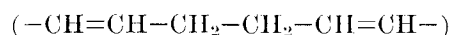
or having both divinylethane and divinylmethane arrangements.[3] However, since no authors have reported that highly unsaturated fatty acids of structure I and II occur together in a fish oil, it suggests that the acids occurring in the same sample will have one of the two types of structure.

It is the purpose of this work to clarify which type of structure would be correct, by analyzing the results obtained by a non-destructive analytical method or the nuclear magnetic resonance technique using methyl esters of highly unsaturated fatty acid concentrates.

The structures of the above two types have hitherto been determined by chemical method whereby isolation of highly unsaturated fatty acid in a pure state was indispensable. Because of readiness in autoxidation and isomerization of highly unsaturated fatty acids, the isolation from fish oil, including various kinds of highly unsaturated fatty acids, is complicated and difficult. Hence, in this work the samples containing various kinds of highly unsaturated fatty acids have been concentrated from fish oils without isolating individual acids, to determine the type of structure by utilizing the nuclear magnetic resonance spectral analysis.

The resonance frequency of the hydrogen atom in the nuclear magnetic resonance spectrum depends

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[1] Present address: Basic Research Laboratory, Toyo Rayon Co., Ltd., Kamakura, Kanagawa-ken, Japan.
[2] An excellent review of the previous works has been published by O. Notevarp in "Fish As Food," edited by G. Borgstrom, Academic Press, Inc., New York, N.Y., 1961, Vol. 1, pp. 260–263.

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