these are incapable of forming adsorbed films on the enzyme surface.

Summary

Experimental evidence from the literature is given to support the hypothesis that the GS_3 which may be present in natural fats cannot be in excess of the quantity which can exist in the fluid state *in vivo*. It is suggested that solid fat is not produced *in vivo* because the mode of action of lipolytic enzymes allows them to form only liquid fats.

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Estimating Carbonyl Compounds in Rancid Fats and Foods[1]

A. S. HENICK, M. F. BENCA,[2] and J. H. MITCHELL JR., Quartermaster Food and Container Institute for the Armed Forces, Chicago, Illinois

THE need for an objective test which will correlate with flavor changes in fats and foods containing fats is apparent to all workers in the field. The only test now in general use, that for peroxide value, is somewhat less than satisfactory especially since the hydroperoxides of fats are generally without flavor or odor. A useful test to correlate with flavor changes, then, should measure some substance, formed during the autoxidation of fat, which is inherently flavored or odorous. Among the several substances in this category, carbonyl compounds seem particularly promising.

Several recent publications (2, 4, 6) report finding carbonyl compounds in the volatile products of autoxidized fats and oils. Although some of these compounds have been qualitatively identified, no quantitative method of universal applicability has been published. The use of 2,4-dinitrophenyl hydrazine derivatives in the colorimetric determination of mono-functional aldehydes and ketones was proposed by Pool and Klose (5), who used a chromatographic separation to eliminate the excess reagents and the derivatives of polycarbonyl compounds. Lappin and Clark (3) described a procedure which also employs the 2,4-dinitrophenyl hydrazones for determining traces of carbonyl compounds in aqueous and alcoholic solutions. Although the latter method was generally inapplicable to fats because the components did not remain in solution throughout the reaction, it was repeatedly observed to give much higher values than did the selective and more reproducible monocarbonyl method of Pool and Klose (5). It was of interest therefore to see whether or not the method of Lappin and Clark could be adapted to use with fats and fat containing foods. The modifications described here permit the quantitative determination of carbonyl compounds in fats and fats contained in foods. The first requirement, a solvent for the fats and the reagents, was fulfilled by benzene. The second requirement, a catalyst which unlike hydrochloric acid usually used would not precipitate as a potassium salt during color development, was met with trichloroacetic acid, the salt of which is soluble in benzene. These modifications and the restandardization necessitated by them gave a procedure universally applicable to fats.

Experimental Procedure

REAGENTS

Carbonyl Free Benzene. Analytical reagent grade benzene is usually sufficiently carbonyl-free as received, but if the blank has an absorbency greater than 0.35 against water at 430 mμ, the benzene can be purified as follows: to one liter of benzene add 5 g. of 2,4-dinitrophenyl hydrazine and 1 g. of trichloroacetic acid; reflux for one hour and then distill through a short Vigreux column.

Carbonyl Free Ethanol. To one liter of ethyl alcohol add 5-10 g. of aluminum granules and 8-10 g. KOH and reflux the mixture for 1 hour. On distilling, discard the first 50 ml. of distillate, and stop the distillation before the last 50 ml. has distilled.

0.05% 2,4-Dinitrophenyl Hydrazine Solution. Dissolve 0.5 g. 2,4-dinitrophenyl hydrazine twice recrystallized from carbonyl-free methanol (which can be prepared in same manner as carbonyl-free ethanol)

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[2] Present address: Custom Chemical Laboratories, Chicago, Ill.

TABLE I

Two-Component Analysis for Hexaldehyde and for Crotonaldehyde

Micromoles									
No.	Hexaldehyde			Crotonaldehyde			Total		
	Calc.	Found	% Error	Calc.	Found	% Error	Calc.	Found	% Error
1	1.203	1.204	0	0.0	0.0	0	1.203	1.204	0
2	.934	.918	−2	.185	.175	−7	1.119	1.093	−3
3	.660	.622	−6	.387	.338	−13	1.047	.960	−9
4	.559	.516	−8	.539	.547	+1	1.098	1.063	−3
5	.225	.218	−3	.760	.753	−1	.985	.971	−1
6	0.0	.003	0	.963	.961	0	.963	.964	0

in one liter of carbonyl-free benzene. This solution is suitable for use for several months.

4.3% Trichloroacetic Acid Solution. Dissolve 43 g. of trichloroacetic acid in carbonyl-free benzene and make to one liter.

4% Potassium Hydroxide Solution. Dissolve 4 g. of potassium hydroxide in 100 ml. of absolute carbonyl-free ethanol with the aid of gentle heating and shaking. Filter the solution through fine glass wool using suction. This solution should be prepared fresh daily.

METHODS OF ANALYSIS

Into a 50-ml. volumetric flask pipette 3 ml. of the trichloroacetic acid solution, 5 ml. of the 2,4-dinitrophenyl hydrazine solution, and 5 ml. of a benzene solution containing the fat to be analyzed. This solution of the fat should be not more than 250×10^{-6} molar in carbonyl. Stopper the flask and heat in a water bath at 60°C. for 30 minutes, then cool to room temperature. This solution is stable for several hours. To develop the color add 10 ml. of the KOH solution, dilute to volume with carbonyl-free absolute ethanol and mix. After exactly 10 minutes read the absorbency at 430 and 460 mμ against a blank prepared in exactly the same manner, substituting 5 ml. of carbonyl-free benzene for the sample solution.

In preparation of samples for analysis, fats and oils are easily dissolved in benzene and 5-ml. aliquots used. Solid foods are ground in a mortar or a mill. Samples are weighed out into glass stoppered centrifuge bottles, benzene is added, the bottle is stoppered, shaken and centrifuged. Five-ml. aliquots of these extracts are then taken for analysis.

Solutions and extracts of fat for this test should be protected from undue exposure to light and air before use to prevent deterioration of existing carbonyl compounds and also further oxidation of the fat which may produce new carbonyl groups. For example, a solution of lard in benzene was permitted to stand 4 hours in diffuse sunlight, after which time the carbonyl value had decreased from 6.88 to 6.36 micromoles per gram. When the test procedure is carried out in less than an hour after preparation of the sample the data obtained are reliable. Fading occurs after the addition of the alkaline ethanol; this fading is a function of time alone, proceeding at the same rate in both light and dark.

STANDARDIZATION OF THE METHOD

In order to express the absorbencies obtained by this method in terms of moles of carbonyl function, the spectral absorbencies of 2,4-dinitrophenyl hydrazine derivatives of several purified carbonyl compounds were measured. In a similar method, using methanol solutions, Lappin and Clark (3) reported that for 17 different saturated monocarbonyl compounds and for methyl phenyl diketone the maximum absorption occurred at 480 mμ and the molar absorbency index,[3] a_M, averaged 27,000 per functional carbonyl group. Pool and Klose (5), in a chromatographic procedure for saturated monocarbonyl compounds reported an a_M of 19,200 at 435 mμ. Using the present method of analysis, the 2,4-dinitrophenyl hydrazones of saturated aldehydes exhibited maximum absorption at 432 mμ, and the a_M was 16,670, while for this derivative of crotonaldehydes, an α,β-unsaturated aldehyde, maximum absorption was at 458 mμ and a_M was 28,100.

Known mixtures of hexaldehyde and crotonaldehyde were prepared and examined for suitability of two-component analysis. The most suitable wave-lengths for this determination are 430 and 460 mμ. At the former wave-length the a_M for the saturated component is 16,000 and for the unsaturated 21,350. At 460 mμ these are 12,450 and 28,100, respectively. When the measurements are made in 1-cm. cuvettes with the Beckman DU Spectrophotometer the analysis can be calculated, using the following equations:

$$\text{Unsaturated} = \frac{3.861\ A_{s\,460} - 3.012\ A_{s\,430}}{0.854}$$

and

$$\text{Saturated} = 3.861\ A_{s\,460} - 2.170\ \text{Unsaturated}$$

An application of this two-component determination to a series of known mixtures is shown in Table I.

Detailed absorption spectra of the mixtures used in Table I were obtained, especially in the regions of the maxima. From these spectra, shown in Figure 1, linear relationships between the mole fraction of unsaturated carbonyl and wave-length of the maximum and absorptivity at the maximum were obtained. These relationships are given in Table II.

A sample of lard, the hydrazones of which exhibited maximum absorption at 440 mμ, was employed to test the reproducibility of the method. Triplicate 1-g. samples were diluted to 50 ml. with benzene, and

[3] Terminology of NBS Circular 484, Spectrophotometry.

TABLE II

Sample	Absorption Maxima of Two-Component Mixtures		
	Mole fraction unsaturated	Wave-length at the maximum mμ	A/micromole at the maximum
1	0.000	435	0.300
2	0.166	439	0.345
3	0.370	444	0.397
4	0.491	447	0.423
5	0.772	453	0.500
6	1.000	458	0.562

$$\text{max} = 23\ f + 435$$

$$\frac{A}{\mu\ \text{mole}} = 0.262\ f + 0.300$$

when f is the mole fraction of α,β-unsaturated aldehyde.

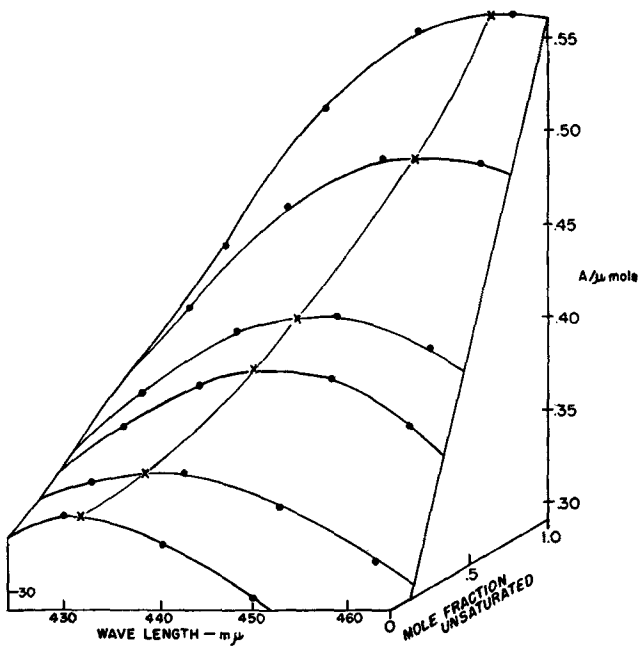

FIG. 1. The absorbency of the 2,4-dinitrophenyl hydrazones of mixtures of saturated and α,β-unsaturated carbonyls.

TABLE III

Replicate Analyses of a Lard

Sample	Weight of Fat g	A_S[a]	Carbonyl Micromoles/g
1a	0.1018	.252	5.90
1b	0.1018	.246	5.76
2a	0.1064	.247	5.53
2b	0.1064	.245	5.49
3a	0.1035	.253	5.82
3b	0.1035	.249	5.73
			5.70±0.164

[a] A_S = Absorbency of the test solution at 440 m/μ.

duplicate 5-ml. aliquots of each solution were analyzed. Measurements were made using a Coleman, Model 14, spectrophotometer employing standardizations from Table II. The results of these analyses, shown in Table III, indicate that at a 95% confidence interval (1) the carbonyl content of the lard examined was 5.70 ± 0.164 micromoles per gram.

A similar test for reproducibility was made using a food product, shoe string potatoes. After grinding and blending, quintuplicate 1-g. samples were extracted by shaking with exactly 20 ml. of benzene. After the residue had settled, 5-ml. aliquots of each extract were analyzed for carbonyl and another 5-ml. aliquot for fat. The fat content of the material was also determined by ether extraction in a Soxhlet extractor and was found to be 35.17%. The results of the analyses described are shown in Table IV, where carbonyl is reported both as that contained in the fat actually extracted with benzene and calculated as that contained in the total fat present in the potato product.

TABLE IV

Carbonyl Content of the Fat of Shoe String Potatoes

Sample	Weight of sample g	Weight of fat in 5 ml. aliquot	Extracted fat %	As	Carbonyl Micromoles/g based on	
					Fat extracted	Fat present[a]
1	1.002	0.088	35.12	0.396	10.71	10.69
2	0.987	0.084	34.04	0.380	10.77	10.44
3	1.044	0.085	32.57	0.410	11.48	10.63
4	0.959	0.093	38.79	0.380	9.73	10.72
5	0.954	0.076	31.87	0.391	12.25	11.09
			34.47		10.99 ±1.29	10.71 ± .33

[a] 35.71% determined by extraction in soxhlet.

It is apparent from Table IV that while shaking with benzene may not be a quantitative method for extracting fat, it is reproducible for extracting carbonyl compounds. The results of the analyses shown in Table IV indicate that at a 95% confidence interval the carbonyl content of the fat of the particular shoe string potatoes used was 10.71 ± .33 micromoles per gram.

APPLICATIONS OF THE METHOD

Two vegetable oils were heated in contact with air at 97.8°C. Periodic sampling was made for peroxide value, saturated and allenic carbonyl content, absorptivity at 268 and 230 mμ, and for flavor change. The data are summarized in Table V.

A prime steam lard was aged in an air oven at 62.5°C. and sampled periodically for both saturated and allenic carbonyl content. The results of these determinations are shown in Table VI.

It can be observed in Tables V and VI that in the vegetable oils examined the rate of increase of the

TABLE V

Analysis of Two Vegetable Oils

Hours of heating 98.7°C.	P.V. me/kg.	Carbonyl, micromoles/g			$E^{1\%}_{1\,cm}$		Flavor[a]
		Sat.	Unsat.	Total	268	230	
Corn Oil							
0	1.3	0.7	8.9	9.5	3.45	4.2	
2	21.1	0.9	11.7	12.6	.....	.....	
4	46.5	3.8	13.9	17.8	3.37	9.5	
6	80.8	7.8	19.6	27.4	.....	.....	
8	111.6	7.4	24.2	32.5	.....	.....	?R
10	275.5	29.0	42.6	71.6	2.85	27.9	R
12	406.7	55.2	78.5	133.7	.....	.....	R
14	560.2	127.9	119.0	246.8	3.09	49.6	R
Soybean Oil							
0	4.3	4.2	3.5	7.6	1.06	2.7	
2	5.1	4.4	3.7	8.0	.....	.....	
4	14.4	6.5	5.6	12.0	.....	.....	
6	30.0	4.0	9.2	13.1	.....	.....	
8	51.8	8.3	12.2	20.4	1.17	8.4	P
10	81.0	9.8	18.2	28.0	.....	.....	P
12	108.7	9.9	26.3	36.2	1.30	13.8	R
14	141.8	17.6	32.7	50.3	1.40	17.0	R

[a] R = Rancid, P = Painty.

TABLE VI

Carbonyl Content in Aged Lard[a]

Carbonyl	Days at 62.5°C.				
	0	0.8	9	14	23
Saturated	6.4	6.7	9.4	9.9	37.7
Unsaturated	1.9	2.2	6.4	6.3	18.3
Total	8.3	8.9	15.8	16.2	56.0

[a] Micromole/gram.

unsaturated carbonyl exceeds that of the saturated while the opposite effect is noticed in the lard. The increase in the carbonyl of these aging vegetable oils parallels the increase in the peroxide, but at lower values. The appearance of rancid flavor in both oils accompanied by similar peroxide and carbonyl values suggests that a relationship exists. A marked increase in the rate of formation of saturated carbonyl after the rancid flavor appears is observed in both oils. Further work is in progress to explain these observations.

Summary

A convenient method for the quantitative determination of the carbonyl compounds in fats and oils is described. The procedure is based upon the formation of the 2,4-dinitrophenyl hydrazones of the carbonyl compounds in the presence of trichloroacetic acid catalyst and the colorimetric determination of the hydrazone compounds in alkaline solution. Standardizations are presented for the simultaneous determination of saturated and allenic carbonyl content from the absorbences at 430 and 460 mμ. Applications of these procedures to edible oils are described, and data are presented showing the carbonyl values in relation to other criteria of change. The problem of correlating changes in the carbonyl content of oils with flavor deterioration is now under investigation.

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The Antioxidant and Antipolymerization Properties of Gossypol, Dianilinogossypol, and Related Materials

W. G. BICKFORD, F. C. PACK, L. E. CASTILLON, and C. H. MACK, Southern Regional Research Laboratory,[1] New Orleans, Louisiana

THE availability from certain other investigations in this laboratory of preparations of gossypol and several related materials led the authors to evaluate these materials for their antioxidant and antipolymerization properties. Mattill (15) and others (11, 18) have reported the antioxidant properties of gossypol, and more recently Hove and Hove (13) have investigated the antioxidant activity of dianilinogossypol. The potential availability of gossypol in amounts exceeding 40,000 tons annually (4, 17) with no current commercial outlet makes gossypol a particularly attractive raw material for investigation.

It was realized at the outset that the toxicological properties of certain of the materials tested were unestablished. However the concentrations at which gossypol has been found to be effective are below reported deleterious physiological levels (3, 9, 12). Dianilinogossypol moreover is said to be physiologically inert (12, 13). Similarly the fact that gossypol and related materials are highly colored even in dilute solution was recognized as a probable deterrent to their use as either antioxidant or antipolymerization agents except in those cases where color is unimportant.

Experimental

Preparation of Materials. The polyphenolic binaphthalene compound, gossypol, was obtained from acetone extracts of cottonseed pigment glands. This pigment was purified by crystallization from diethyl ether-light petroleum naphtha mixture. The yellow dog-toothed prisms of gossypol (1, 6) melted at 184°C.

Dianilinogossypol was prepared according to the method of Clark (8). It was recrystallized from chloroform and yielded orange rectangular plates (2) m.p. 300°C.

The combination products of gossypol-glycine and gossypol-urea were prepared by combining equal parts by weight of gossypol and urea and nine parts by weight of gossypol with one part by weight of glycine. The ingredients were combined in aqueous sodium hydroxide solution of pH 11. The pH of the mixtures was lowered to 7 by addition of hydrochloric acid, and the solutions were lyophilized. The yellow, dried combination products were water-soluble and contained sodium chloride (5, 7).

Gossypolaminobenzenethiol was prepared by dissolving one g. of gossypol in 20 ml. of diethyl ether. To this was added 4 ml. of 2-aminobenzenethiol. The product was washed with diethyl ether and dried. It was repeatedly recrystallized from hot benzene until further recrystallization did not increase the melting point. The orange boat-shaped crystals melted at 270° C. The formation of gossypolaminobenzenethiol might be expected to result from the reaction of 2 moles of 2-aminobenzenethiol with one mole of gossypol, producing a Schiff's base similar to dianilinogossypol. But the analytical data for gossypolaminobenzenethiol do not support this assumption.

Anal. Calc'd for $C_{30}H_{40}O_6S_2N_2$: C, 68.9; H, 5.46; N, 3.83; S, 8.74.

Found: C, 72.5; H, 5.8; N, 4.02; S, 3.31; ash, 0.21.

[1] One of the laboratories of the Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U. S. Department of Agriculture.