Analysis of Fat Deterioration—Comparison of Some Photometric Tests

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ABSTRACT AND SUMMARY

Eight photometric methods (e.g., measurement of the diene absorption, ferrous isothiocyanate test, thiobarbituric acid value test, anisidine value, Kreis test) for analysis of the oxidative deterioration of fats were compared with respect to sensitivity against autoxidized methyl linoleate and methyl linolenate. The ferrous isothiocyanate test was found to be the most sensitive method followed by the measurement of diene absorption. For assessment of the specificity, highly autoxidized methyl linoleate was separated by column chromatography into the following classes of compounds: volatile carbonyl compounds, monohydroperoxides, and polar peroxides-1 and -2. The influence of each class of compounds on the results of the eight tests was determined.

INTRODUCTION

Numerous simple photometric tests for analysis of the oxidative deterioration of food lipids are reported in the literature (1,2). A comparative study of the sensitivity and specificity of these various methods is of interest. We have therefore determined the relative sensitivities of eight methods against low (POV $<$ 600) and high POV $\sim$ 3000) oxidized linoleic and linolenic acid methyl ester. For assessment of the specificity, highly oxidized methyl linoleate was separated into four classes of compounds. The influence of each class of compounds on the results of the eight tests was determined.

EXPERIMENTAL PROCEDURES

Methyl esters of linoleic and linolenic acids (ca. 100 mg each, about 98% purity) were autoxidized under UV light at room temperature. Silica Gel 60 (Merck, Art. Nr. 7734; 0.063-0.2 mm) was washed with HCl and conditioned for 2 hr in a rotary evaporator (50 C, 20 Torr) according to Esterbauer (3). Linoleic acid hydroperoxides (LOOH) were prepared by the oxidation of linoleic acid with soya lipoxygenase and purified by thin layer chromatography (TLC) (4). A mixture of 9 parts per volume cyclohexane (C) and 1 part per volume diethyl ether (D) was used for dilution of the autoxidized fatty acid methyl esters and the linoleic acid hydroperoxides. Colour Developer 3: {N-2- [N-aethyl-N-(4-amino-3-methylphenyl)-amino} -aethy- methansulfonamidsesquisulfate, was from Merck (Darmstadt, Germany). Carbonyl free solvents were prepared by the methods of Schwarz and Parks (5) and Gaddis et al. (6).

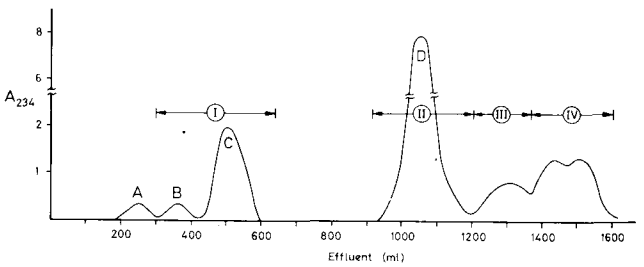

FIG. 1. Chromatographic separation of autoxidized methyl linoleate with aldehydes added. Sample: About 100 mg methyl linoleate, 1.5 mg 2.4-decadienal, and 3 mg hexanal dissolved in 3.5 ml cyclohexane:diethyl ether (9:1, v/v). Column: Silica Gel 60 (2 x 100 cm). Elution at 4 C with 750 ml cyclohexane:diethyl ether (9:1, v/v) and 1000 ml of a linear gradient of cyclohexane:diethyl ether (9:1 → 3:7, v/v). Identified compounds were: A—methyl linoleate, B—hexanal, C—2.4-decadienal, D—monohydroperoxides of methyl linoleate. The following fractions were collected: I—volatile carbonyl compounds, II—monohydroperoxides, III—polar peroxides-1, IV—polar peroxides-2.

Sample

About 100 mg of the autoxidized fatty acid methyl ester (POV $<$ 600) were dissolved in 50 ml C/D. More oxidized esters (POV $>$ 2000) were dissolved in 200 ml C/D.

Methods

The details of the various methods were followed as given in the references cited against each method.

UV-absorption: After diluting 0.5 ml of the sample with 3 ml methanol, the absorbances at 234 nm (diene) and 270 nm (triene) were measured.

α,α'-Diphenyl-β-picrylhydrazyl reaction (DPPH) (7): To the sample (0.6 ml), 3 ml of the reagent was added diluted to 3 ml with C/D. The absorption of the solution was measured at 517 nm against the reagent blank.

Fe test (8): A mixture was made from 0.1 ml sample, 4.85 ml benzene:methanol (7:3, v/v), 0.1 ml water, and 3.6 μmol $FeSO_4$ dissolved in 0.02 ml 3.6% HCl. Thirty seconds after the addition of the $FeSO_4$, 0.02 ml 30% KSCN was added. The development of the color and measurement of its intensity were followed as described (4).

Thiobarbituric acid value (TBA) (9,10): The sample (2.5 ml) was diluted to 6 ml with C/D and 1 ml ethanol, and 3 ml TBA pipetted into it. After warming to 60 C (30 min) the absorbances at 452 nm and at 530 nm were read against a reagent blank.

Anisidine value (11): The sample (2.0 ml) was mixed with 4 ml 1.5% solution of trichloroacetic acid in ethanol and 4 ml 0.25% p-anisidine solution in ethanol. After warming to 60 C (60 min) the absorbance at 400 nm was read against a reagent blank.

Kreis test (12): The sample (2 ml) diluted with C/D to 3 ml, was mixed with 3 ml 60% solution of trichloroacetic acid in glacial acetic acid and 1 ml of a solution of 1% phloroglucin in glacial acetic acid (w/v). After 15 min at 37 C the absorbance at 540 nm was read against a reagent blank.

Heptanal value (13): The sample (2.0 ml) was treated by shaking with 1 ml 0.5% 2.4-dinitrophenylhydrazine dissolved in benzene, 1 ml methanol, and 0.5 ml 60% acetic acid in benzene. After separation of the excess reagent by filtration through a cation exchanger, the absorbance at 366 nm was read against a reagent blank. The total volume was 10 ml.

F-3 test (14): The sample (0.1-0.5 ml) was diluted to 5 ml with a mixture of acetone:$CHCl_3$:glacial acetic acid (5:3:2, v/v/v). Twenty minutes after the addition of 0.1 ml of the reagent, which contained 300 mg Colour Developer 3 (Merck) per 27 ml, the absorbance was measured at 510 nm against a reagent blank.

Iodometric peroxide determination (15): A micro burette was used.

TABLE I

Determination of Relative Sensitivities (R)

Method		Reference	Sample[a] (ml)	Volume[b] (ml)	Absorbance measured	Absorbance calculated[c]	R[d]
1. UV absorbance	234 nm		0.5	3.5	0.79	0.55	1
	270 nm		0.5	3.5	0.09	0.063	0.1
2. DPPH		(7)	0.6	6.0	0.275	0.275	0.5
3. Fe test		(8)	0.1	5.1	1.04	5.2	9.4
4. TBA	452 nm	(9,10)	2.5	10,0	0.068	0.03	<0.1
5. Anisidine value		(11)	2.0	10.0	0.32	0.16	0.3
6. Kreis test		(12)	2.0	7.0	0.20	0.07	0.1
7. Heptanal value		(13)	2.0	10.0	0.04	0.02	<0.1
8. F-3 test		(14)	0.5	5.1	0.17	0.17	0.3

[a]Sample: 124 mg autoxidized methyl linoleate (POV 475) in 50 ml cyclohexane:diethyl ether (9:1, v/v).

[b]Volume after dilution and addition of the reagents.

[c]The absorbances were calculated at a dilution of 1:10.

[d]The values of absorbances calculated were divided by the normalized diene abosorption value (0,55) to calculate the relative sensitivities, R.

TABLE II

Comparison of the Relative Sensitivities[a]

Method		Linoleic acid hydroperoxide[b] absorbance	R	Methyl linoleate[c] POV 475 absorbance[e]	R	Methyl linoleate[c] POV 2750 absorbance[e]	R	Methyl linolenate[d] POV 450 absorbance[e]	R	Methyl linolenate[d] POV 3300 absorbance[e]	R
UV absorbance	234 nm	7.42	1	0.55	1	6.48	1	0.91	1	4.27	1
	270 nm	0.07	<0.1	0.063	0.1	0.39	<0.1	0.29	0.3	1.21	0.3
DPPH		2.15	0.3	0.275	0.5	1.08	0.2	0.91	1	2.31	0.54
Fe test		26.4	3.6	5.2	9.4	35.6	5.5	5.7	6.3	9.8	2.3
TBA	452 nm	0.07	<0.1	0.03	<0.1	0.36	<0.1	0.42	0.46	0.9	0.2
	530 nm	---	---	---	---	0.18	<0.1	0.9	1	2.0	0.5
Anisidine value		0.12	<0.1	0.16	0.3	1.3	0.2	0.75	0.8	1.8	0.4
Kreis test		--	---	0.07	0.1	1.04	0.2	0.10	0.1	0.34	0.1
Heptnal value		---	---	0.02	<0.1	2.92	0.5	0.04	<0.1	0.55	0.1
F-3 test		1.65	0.2	0.17	0.3	0.5	<0.1	---	---	--	---

[a]The relative sensitivities (R) were calculated as shown in Table I.

[b]Linoleic acid hydroperoxides (92.6 mg) dissolved in 100 ml C/D.

[c]Methyl linoleate POV 475 (124 mg) and POV 2750 (70 mg) dissolved in C/D as described in the experimental section.

[d]Methyl linolenate POV 450 (110 mg) and POV 3300 (117 mg) dissolved in C/D as described in the experimental section.

[e]The absorbances are normalized to a dilution of 1:10 (see Table I).

Chromatographic separation of the autoxidized fatty acid methyl ester: About 100 mg of autoxidized methyl linoleate, dissolved in 3.5 ml C/D, were applied to a silica gel column (2 x 100 cm) with cooling jacket maintained at 4 C. The elution was made with 750 ml C/D and 1000 ml of a linear gradient of C/D (9:1 → 3:7, v/v) at 33 ml/hr. The volume of each fraction was 10 ml. The compounds were detected in the eluate by measuring the absorbances at 220 nm and 234 nm. The tubes containing volatile carbonyl compounds, monohydroperoxides, polar peroxides-1 and -2 (Fig. 1) were combined. Each class of substances was tested with eight methods.

Analysis of monohydroperoxides: The monohydroperoxide fraction was separated from autoxidized linoleic acid methyl ester on a chromatographic column. After reduction with $NaBH_4$ and hydrogenation with Pd/C the determination was made mass spectrometrically as described earlier (16).

RESULTS

Comparison of the Sensitivity

The autoxidized samples, whose POV had been determined by iodometric titration, were dissolved in C/D and after the addition of the reagents, were diluted to the volumes given in Table I for measuring the absorbances. For comparison, the measured absorbances were calculated at the same dilution, 1:10, and related to the diene absorption (relative sensitivity R 1.0). Table I shows the procedure with autoxidized linoleic acid methyl ester as an example.

TABLE III

Sensitivity of the Iodometric POV Determination in Comparison with the Fe Test and Diene Absorption Measurement

Method	100 nmol LOOH
Iodometric titration (15)	consume 4.5 µl 0.1 N $Na_2S_2O_3$
Fe test (8)	give $E^{1\ cm}_{505} = 1.786$
Diene absorbance	give $E^{1\ cm}_{234} = 0.714$

In Table II the investigated samples, the absorbances measured and normalized to a dilution of 1:10, and the calculated R values are listed. The autoxidized samples of methyl linoleate and methyl linolenate and TLC purified LOOH were analyzed. In order to show the influence of the oxidation state on the methods, both low and high oxidized esters were tested.

In Table II the high sensitivity of the Fe test in comparison with the diene absorption and the other methods is remarkable. The differences are indeed dependent upon the type of fatty acid and its oxidation state but they nevertheless appear clearly with every sample. Thus, against the samples of linoleic acid methyl ester and the low autoxidized linolenic acid methyl ester, the Fe test is about 5-9 times more sensitive than diene absorption measurement.

The autoxidation of linolenic acid is probably accompa-

TABLE IV

Chromatography of Methyl Linoleate (POV 2900): Percent Contribution[a] of Four Separated Classes of Compounds to the Analysis Values Resulting from the Eight Photometric Methods

Method		Volatile carbonyl compounds	Monohydroperoxides	Polar peroxides-1	Polar peroxides-2
1. UV absorbance	234 nm	0	76	6	18
	270 nm	34	0	38	28
2. DPPH		0	71	4	25
3. Fe Test		0	73	10	17
4. TBA	452 nm	0	44	12	44
	530 nm	0	0	31	69
5. Anisidine value		25	25	17	33
6. Kreis test		14	17	37	32
7. Heptanal value		44	10	36	10
8. F-3 test		0	77	4	20

[a]The percent contribution was calculated by dividing the A_c-value of the class of compounds through the Σ A_c-values of the four classes of compounds.

$$A_c = \frac{A \cdot V_c}{V_s}$$

A_c = total absorbance of the class of compounds;
A: absorbance measured;
V_s: volume of the sample in the photometric test;
V_c: total elution volume of the class of compounds.

TABLE V

Chromatography of Methyl Linoleate (POV 2900) Spiced with Aldehydes[a]: Percent Contribution of Four Separated Classes of Compounds to the Analysis Values Resulting from Four Photometric Methods

Method	Volatile carbonyl compounds	Monohydroperoxides	Polar peroxides-1	Polar peroxides-2
1. TBA 452 nm	81	6	3	10
2. Anisidine value	57	26	7	10
3. Kreis test	58	13	12	17
4. Heptanal value	90	3	5	2

[a]Before chromatographic separation 1.5 mg 2.4-decadienal and 3 mg hexanal were added to 90 mg autoxidized methyl linoleate.

[b]The calculation of the percent contribution is described in Table IV.

nied by side reactions to a greater extent than is that of linoleic acid resulting in the formation of a wide range of products. Methods, such as DPPH, TBA, and anisidine value therefore react more sensitively to the autoxidation of linolenic acid than they do to linoleic acid (Table II). The Fe test, but not the diene measurement, of the highly oxidized methyl linolenate shows a significantly lower analysis value than that of the corresponding oxidized methyl linoleate (Table II).

Evidently a considerable proportion of the hydroperoxides is broken down in the autoxidation of linolenic acid methyl ester and the diene system remains. As a result, in highly autoxidized methyl linolenate the R value of the Fe test does not differ much from that of the diene absorption as in low oxidized samples.

In Table III the results of an iodometric titration (micro method) of LOOH, a measurement of the diene absorption, and the Fe test are compared. The sensitivity of iodometric method does not compare with the Fe test even when diluting sodium thiosulfate ten times.

Selectivity of the Methods

The differing sensitivities can be attributed to the fact that the classes of compounds resulting from the oxidative deterioration of the fatty acids are picked up with varying intensities by the eight methods separately. In order to get an insight into this, a model experiment was carried out.

Methyl linoleate was autoxidized up to a high POV = 2900 and separated chromatographically into volatile carbonyl compounds, monohydroperoxides, and polar peroxides-1 and -2. The proof that the major peak (Peak II in Fig. 1) is made up of a mixture (1:1) of 9-hydroperoxyoctadeca-10,12- and 13-hydroperoxyoctadeca-9,11-diene acid methyl esters were confirmed after reduction of the hydroperoxy groups, hydrogenation of the diene system, and through mass spectrometric analysis of the resulting hydroxystearic acid methyl esters.

The percentage contribution of each of the four chromatographically separated classes of compounds to the analysis values of the eight tests were determined (table IV). Accordingly, the monohydroperoxides appearing as the main class are favored in the measurement of the UV absorption at 234 nm, the DPPH, Fe, and F-3 tests. Against this, the TBA value (530 nm) favored the polar peroxides. The values given by the determination of the UV absorption at 270 nm, of the anisidine method, the Kreis, and the heptanal tests are more or less affected by the volatile carbonyl compounds.

The main volatile carbonyl compounds resulting from the autoxidation of linoleic acid are hexanal and 2,4-decadienal (17). The concentration of these compounds, which could be very important in the formation of a rancid off-flavor are very small even in a highly autoxidized sample. Because of this, autoxidized linoleic acid methyl ester, spiced with hexanal and 2,4-decadienal was chromatographed and, as described above, the contribution of each of the four classes of compounds to the analysis values was estimated. The results confirmed that the volatile carbonyl compounds are not noted in the UV absorption at 234 nm, the DPPH, Fe and F-3 tests. Table V contains only the chemical analysis methods in which the reaction with this class of compounds takes place. Volatile carbonyl compounds show up in the TBA (452 nm) and especially in the heptanal value (Table V). Although the anisidine value and the Kreis test signal the presence of carbonyl compounds,

the measured absorbances nevertheless are influenced by the three peroxide fractions.

DISCUSSION AND CONCLUSIONS

Should hydroperoxides be formed during storage of a fat containing foodstuff and should these only break down slowly, then the Fe test is found to be the most sensitive photometric method for detection of autoxidation. DPPH and the F-3 tests do also show mainly peroxides but are considerably less sensitive than the Fe test. The Fe test is also superior to an iodometric titration. With a Wheeler titration, 10 μmol/ml peroxide can be determined (1) and a micro method raises the level to 10 nmol/ml peroxide (15). However, the Fe test with a limit of 1-5 nmol/ml peroxide is still more sensitive. The only drawback of the Fe test is the unexplained stoichiometry (4).

Due to an interaction with other substances, the peroxides in some foodstuffs break down very quickly. The breakdown of peroxides from linoleic or linolenic acid would give among others, products with a remaining diene system. The measurement of the diene absorption at 234 nm in such cases is the most sensitive method of detecting the beginning of a fat oxidation.

An analysis of the carbonyl compounds formed by autoxidation is problematic in the presence of peroxides. The heptanal value was found to be the most selective although the use of acids also leads to the formation of additional carbonyl compounds from peroxides. In this way the results are misleading considerably due to the side reactions, because only traces of carbonyl compounds are formed in a rancid fat.

The anisidine value and the Kreis test are not suitable for the detection of fat oxidation. These two methods are insensitive and totally unspecific.

Numerous variations in the procedure of the TBA test are suggested in the literature (2). To increase the sensitivity, an addition of Fe^{+3} ions (19,20) is recommended. The reason for higher values is due to the breakdown of peroxides to products which react with TBA (9,21). Besides this, the possibility cannot be excluded that under drastic reaction conditions the sample is further autoxidized or that fat accompanying substances influence the TBA test (19,22). Since in this case there is no real increase in sensitivity, a procedure for the TBA reaction (9,10) was chosen in which acid and Fe^{+3} ions were not present.

As already noted (23) and recently explained (24), the autoxidation products of linoleic acid react over a considerably smaller range with TBA than those of linolenic acid and is also confirmed by our results.

Our results further show that even for autoxidized linolenic acid the TBA test is less sensitive than the Fe test or the measurement of the diene absorption.

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