The Reaction of Picric Acid with Epoxides. II. The Detection of Epoxides in Heated Oils[1]

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Abstract

A colorimetric method, based upon the reaction of the oxirane group with picric acid, was used to determine the epoxide content of heated vegetable oils. The picration method is particularly suitable for measuring small quantities of epoxide because it is much more sensitive than the common titrimetric methods, and it is not subject to interference from cyclopropene, conjugated dieneols, or α,β-unsaturated carbonyls.

Thin-layer chromatography was used to separate mixtures of picrated, epoxidized methyl esters. Separation of *cis*- and *trans*-methyl epoxystearate, methyl epoxyoleate, and methyl diepoxystearate in a mixture of these four esters was achieved in this manner. The presence of saturated *cis*- and *trans*-epoxystearates and unsaturated epoxides was demonstrated in heated vegetable oils.

Introduction

IN A PREVIOUS PUBLICATION (1), a colorimetric method was described for the analysis of epoxides by reaction with picric acid. Evidence was presented which demonstrated that the principal reaction product was an hydroxypicryl ether. For the purpose of quantative measurements, this derivative was treated with a base which resulted in the development of an orange color. Since the base-treated picrate exhibits an absorption at about 490 mμ, in a region where the interference from picric acid is negligible, this wavelength was selected for the measurements.

Our interest in exploring the value of picration arose from a need to measure the very small quantities of epoxides present in heated fats. The accepted methods, based upon the addition of hydrogen halides to the oxirane group (2,3), lack specificity. Besides reacting with the oxirane moiety, hydrogen halides also attack to some degree α,β-unsaturated carbonyls and conjugated dieneols, which are known to form when oils are heated. Sometimes this interference can be minimized, but not completely eliminated, by titrating at 3C. But then a stoichiometric addition to oxirane is not certain. Skau has used this stepwise technique to detect epoxides in the presence of cyclopropenes known to be present in cottonseed oil and to interfere with the usual oxirane titration (4).

In addition to lack of specificity, hydrogen halides do not react quantitatively with some *trans*-epoxides (5) which may form during heating. Furthermore, these methods do not have a very high sensitivity, requiring at least 0.1 mmole of oxirane, about 0.2% oxirane oxygen, in the oil. The amount of epoxide in heated oils is often below this level.

The Jay method (3), which depends upon generating HBr in situ from tetraethylammonium bromide and perchloric acid, has also been examined and found to be subject to the same interferences as the direct HBr titration method (2). The Morris and Holman procedure (6) based upon infrared spectrophotometry of the halohydrin is somewhat involved and does not give the same result with both *cis* and *trans* epoxides.

A major concern in applying the picration method to heated fats was to establish that it did not have similar failings with regard to various interfering substances. In addition, it was desired to separate and identify the various types of epoxides which form during the heating of vegetable oils.

Experimental Procedures

Heating Experiments

Pure olive oil, cottonseed cooking oil, and soybean salad oil were heated in bulk in open two-liter beakers inserted into cylindrical heating mantles. Each beaker contained about 1600 g of oil and was slowly stirred by magnetic agitation so as to prevent vortex formation. At regular intervals 15–20 g aliquots were removed for peroxide, oxirane, and carbonyl assays. Each of the three oils was heated at 80C (42 days), 150C (9 days) and 250C (22 hr).

At the end of the heating period, the oils were allowed to stand overnight at room temperature, and then each was analyzed for free fatty acids (7), peroxide value (8), carbonyl content (9), and total epoxide level.

Analysis for Epoxides

An evaluation was made of the picration method (1) for specificity in the measurement of epoxides in heated oils. The method has been modified slightly for application to heated oils. A sample containing 0.04–0.4 meq of oxirane is weighed into a 10 ml volumetric flask and dissolved in about 5 ml of ether. After adding 2.0 ml of 0.25 M picric acid solution (in 95% ethyl alcohol), the mixture is made to volume with ether, mixed and allowed to stand at room temperature for 24 hr. A 1.0 ml aliquot is removed, pipeted into a 50 ml volumetric flask, made basic with 10.0 ml pyridine and diluted to volume with 95% ethyl alcohol. The optical density of solution is read at 490 mμ within 15 min of dilution. After subtracting the blank value, the oxirane concentration is computed as outlined in Figure 1. It is necessary to select the appropriate standard curve, based upon the type of epoxide present in the oil. Although Beer's Law holds in every case when optical density is plotted vs. concentration, the slope of the curve is different for each epoxide (1). See Results and Discussion for further details.

Thin-Layer Chromatography

Picration of the Heated Oils. Ten grams of the heated oils was transesterified by refluxing for 30 min with 130 ml of 0.02 N sodium methoxide in methyl alcohol (10). Aliquots (20 ml) of these solutions were pipeted into 100 ml volumetric flasks and were made to volume with a 0.083 M solution of picric acid in ethyl ether. After mixing, the solution was allowed to stand overnight at room temperature.

One may effect a preliminary concentration step

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$$C = \frac{0.05\ R}{m}$$
$$= 0.071\ R \text{ for CSO \& SBO}$$
$$= 0.090\ R \text{ for OO}$$

Where: O.D. = Oxirane Conc. (meq/100g oil).

$$R = \frac{O.D. \times 100}{\text{Sample wt.}}$$

(Where: O.D. = optical density @ 490 mμ).
m = Slope of standard curves.

FIG. 1. Calculation of oxirane concentration by picric acid method.

when the epoxide level is low. This is done by removing the methyl alcohol from the trans-esterification mixture by suction under nitrogen at 50C and partially replacing it with 95% ethyl alcohol. The latter is used since it has been found to give a greater conversion of the epoxides to picrates.

Thin-Layer Chromatography. The TLC work was done with Silica Gel G mostly using 250 μ plates (Analtech, Inc., Wilmington, Delaware). The developing solvent was a mixture of 20–40C petroleum ether/ethyl ether/acetic acid (60/40/1, respectively). Separation of the picrates of *cis*-methyl epoxystearate (I) *trans*-methyl epoxystearate (II) and methyl epoxyoleate (III)—also called methyl vernolate—was achieved by TLC on argentated plates. The plates were dipped into a saturated solution of $AgNO_3$ in methanol and air dried prior to use. The picrated derivatives of the model epoxides are colored: I and III are orange; II and methyl diepoxystearate (IV) are deep yellow. These colors are greatly intensified when the TLC plates are exposed to ammonia fumes so that the equivalent of 10 μg of epoxide can be seen with little difficulty.

In those oils where the epoxide concentration was low, preparative TLC was used to concentrate the picrates. In this case, II is best separated from the others on an unargentated plate. Thus, 1–1.5 ml of transesterified oil solution from (a) above was manually streaked on a 20 × 20 cm plate and developed at 5C. After development, the plate was exposed to ammonia fumes; the picrate streaks were scraped into aluminum foil, transferred to small beakers and extracted with a 1–2 ml methyl alcohol. The silica was filtered off and the volume of the alcohol was reduced to 0.2–0.5 ml at room temperature under a stream of nitrogen. A 25–50 μl aliquot of the picrate solutions was then spotted on a $AgNO_3$ treated plate which was developed at room temperature. The results of these experiments appear in the following section.

Results and Discussion

Interfering Substances

The interference by substances mentioned in the first paper (1) was studied in some detail. Table I shows a comparison between results with the standard HBr titration, the Jay method, and picration when applied to some of these. Cyclopropene acids (sterculic and malvalic) present in cottonseed oil were titratable by both methods but gave no color development on picration. Sterculia seed oil, which contains about 50% sterculic acid, gives a much larger response with both titration methods, but again shows no color development with picric acid. Another moiety, known to add acid halides, is the conjugated dienol structure. Methyl dimorphecolate (methyl 9-hydroxy-10:12-octadecadienoate) does not interfere with the picric acid method.

TABLE I
Interfering Substances

Sample	% Oxirane		
	HBr titration	Jay method	Picration
Cottonseed oil	0.01	0.03	0.00
Sterculia seed oil	2.66[a]	2.72	0.00
Methyl dimorphecolate[b]	2.64[c]	1.1[c]	0.00
Acrolein	1.2[c]	>0[c]	0.00
Crotonaldehyde	1.1[c]	>0[c]	0.00

[a] Titrated at 55 ± 5C. [b] Estimated purity 80%. [c] Unstable end point.

TABLE II
Carbonyl Interference

Time after addition of base (min)	Optical Density[a] (3 hr reaction time)[c]				
	2, 4-Hexadienal				Mesityl oxide
	1% NaOH	0.2% NaOH	0.1% NaOH	10% Pyridine	1% NaOH
1	0.075	0.020	0.017	0.014	0.043
2	0.115	0.026	.....	0.014	0.048
3	0.162	0.032	.....	0.014	0.052
5	0.240	0.045	0.025	0.014	0.060
12	0.415	0.088	0.041	.....	.....
15	0.470	0.110	0.050	.....	0.090
30	0.632[b]	0.185	0.091	0.014	0.106
60	0.730[b]	.....	.....	0.012	0.118
24 (hr)	0.305	?[b]	?[b]	0.014	0.088

[a] Solutions = 0.020 M in carbonyl content and 0.050 M in picric acid. [b] Cloudy. [c] Results the same from 2–48 hr.

Unsaturated carbonyls also react with HBr. The reaction is not stoichiometric and is gradual, thus giving uncertain end points. This difficulty was avoided when the picric acid method was applied to acrolein and crotonaldehyde. A further investigation, however, showed that certain carbonyls react with picric acid in the presence of base. This reaction is probably similar to the one used to detect steroid carbonyls (11). As Table II shows, reaction occurs when the mixture is made alkaline in order to develop the hydroxypicryl ether color; it is dependent on the strength of the base and is virtually eliminated by using pyridine.

Quantitation

In Table III are shown the results of free fatty acid, peroxide value, and carbonyl determinations (9) on oils which had been heated at 80C, 150C, and 250C. These values are typical for oils which have been heat-abused in air over prolonged periods. Hydroperoxides accumulate in the oils held below 100C, but they decompose to form secondary oxidation products at the higher temperatures. At the low temperature (80C) unsaturated carbonyls predominate, whereas the saturated ones appear to form more rapidly at the higher temperatures.

Table IV reports the epoxide content of these same oils as measured by titration using HBr directly and formed in situ, and by the picration method. When pyridine is used to develop the color, the resulting values are consistently lower than when NaOH is used, confirming that the interference of carbonyls can be drastically reduced or eliminated. Except for the 80C heated oils, the results of the titration methods at 3C are quite close to those obtained by picration. Besides requiring a much larger sample size (5–10 g vs. 0.2–0.5 g) these methods show drifting end-points and precision suffers thereby. Furthermore, a considerable amount of time is necessary to achieve a stable end-point (one which does not change for 30 sec) when heated oils are titrated.

The values in Table IV have been computed assuming that olive oil contains only saturated monoepoxides; i.e. using the curve for methyl epoxystearate; and that soybean and cottonseed oil contain only mono-unsaturated epoxides (vernolic and coronaric acids). Since, as Table V shows, this is not correct, the oxirane values obtained by picration include the error inherent in this assumption. As a

TABLE III
Free Fatty Acids, Peroxide and Carbonyl Values in Heated Oils

Sample	% Free fatty acids[a]			Peroxide value[b]			Carbonyl value[b]		
	80C	150C	250C	80C	150C	250C	80C	150C	250C
Olive oil	2.85	0.27	0.49	175	1.3	4.5	79.5U 39.3S	36.8U 41.7S	27.5U 37.8S
Cottonseed oil	0.55	0.08	0.33	128	2.8	2.5	65.4U 10.4S	55.4U 26.2S	16.5U 41.7S
Soybean oil	0.57	0.07	0.31	127	3.0	3.1	73.1U 13.7S	42.1U 17.7S	23.2U 51.3S

[a] As % oleic acid. [b] Meq/kg. U = Unsaturated. S = Saturated.

strict quantitative method, therefore, picration leaves much to be desired, since it depends on knowing beforehand the nature of the oxirane moiety being attacked. It can be of value, however, where sample size and/or large quantities of interfering substances make the titrimetric methods prohibitive.

Separation and Identification of Epoxides

Of more significance than the *potential* application of the picration technique as a quantitative method, is its possible use for the qualitative identification of epoxides in heated fats. As Figure 2 shows, it would be difficult, if not impossible, to detect and identify epoxides by direct TLC of the transesterified heated oils. Besides the normal difficulty in detecting the low levels present, the identification is complicated by the poor resolution of the three monoepoxides (I–III) on the TLC plate. Argentation separates III from I and II, but there seems to be no satisfactory way of separating the *cis*- from the *trans*-methyl epoxystearate. Figure 3 shows how picration improves this situation. The model epoxide spots (unpicrated) in this plate were made visible by exposing to iodine vapors. The *trans*-epoxide (II) spot is barely visible when the plate is withdrawn from the acidic environment of the development tank; on drying it becomes yellow. After standing, especially if exposed to ultraviolet light, the spot becomes yellow-green and shows a blue-green fluorescence. This color change, together with its R_f, which is intermediate between that of I and III helps in its identification.

In contrast, the picrate spots of I and III are orange, both during and after development; and their color is quite stable on standing. The color of the spots can be greatly accentuated by making the plate alkaline. This can be done readily by exposing the plates briefly to pyridine vapors or ammonia fumes. This enhancement of color makes the identification of 10 μg of picrated epoxide quite easy. Twice this amount may be necessary to detect epoxide on an argentated plate because of the normally darker background. The deepening of the colors is instantaneous; they fade with time but can be revived by a subsequent exposure. As is evident from Figure 3, picration of model epoxides results in more than one

TABLE IV
Oxirane Determinations in Heated Oils

Heating conditions	Oil	Meq oxirane/100 g oil		
		Picration[a]	HBr method @ 3C[b]	Jay method @ 3C[c]
80C (42 days)	OO	18.23	21.27	24.55
	CSO	7.68	14.65	21.70
	SBO	8.64	17.13	25.14
150C (9 days)	OO	7.63	7.71	6.11
	CSO	5.20	7.58	7.93
	SBO	3.42	4.61	6.84
250C (22 hr)	OO	5.57	0.75	3.8
	CSO	2.52	0.50	2.4
	SBO	3.43	0.95	3.8

[a] Color developed with 20% (v/v) pyridine. [b] Reference 24. [c] Reference 25

TABLE V
Characteristics of a Typical Preparative TLC Plate

Temperature	5C (in the refrigerator).
Time of development	60 min
Solvent travel	17.5 cm
Picric acid band	2–3 cm wide; yellow; starting at the origin.
Band 1 (*cis*-epoxide)	2–3 mm wide; reddish orange; R_f 0.55.
Band 2 (*trans*-epoxide)[a]	1–1.5 cm wide; yellow; R_f 0.65.
Bands 3 and 4 (unknown)	Each 1–2 mm wide; very faint yellow; R_f 0.3 and 0.8.

[a] Fluoresces blue-green on standing.

TLC spot. The diepoxide (IV) shows at least five: two major and three minor spots. The other model epoxides also show fainter yellow spots. Whether the smaller spots are due to isomerism or side reactions of the picric acid with the fatty epoxides is not clear. What is certain is that better than 80% of the total colored material is present in the main picration spots. This has been ascertained by eluting the spots with methanol and measuring absorption of their basic alcohol solutions at 490 mμ. Because of this preponderance, our attention has focused on these main spots.

The Epoxides in 80, 150, and 250C Heated Oils

The appearance of these main spots has been taken as evidence for the presence of epoxides in the nine heated oils. When the oxirane content is substantial, as in the case of the 80C heated oils, the presence of the most abundant picrates can be identified with relative ease by spotting 40–100 μl of picric acid-treated oil solution. Figure 4 illustrates what is seen when picric acid-treated, 80C heated oil is placed in a TLC plate along with model epoxypicrates. Noteworthy here is the fact that the picrate of methyl coronarate (methyl *cis*-9:10-epoxy-*cis*-12:13-octadecenoate), the positional isomer of methyl vernolate (III), has the same R_f as the latter, hence cannot be distinguished from it by this method. The streak-

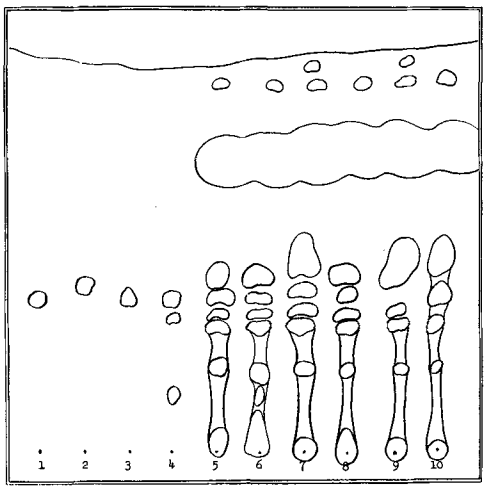

FIG. 2. TLC of model epoxides and 15–250C heated oils: 1) methyl epoxystearate; 2) *trans*-methyl epoxystearate; 3) methyl epoxyoleate; 4) 1–3 + methyl diepoxystearate; 5) 150C olive oil; 6) 150C cottonseed oil; 7) 150C soybean oil; 8) 250C olive oil; 9) 250C cottonseed oil; 10) 250C soybean oil.

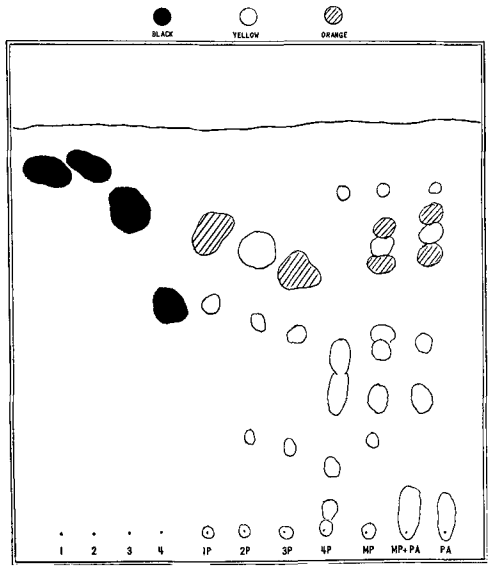

FIG. 3. TLC of model epoxides before and after picration ($AgNO_3$): 1) *cis*-methyl epoxystearate; 2) *trans*-methyl epoxystearate; 3) methyl vernolate; 4) methyl diepoxysterate; *M*, mixture; PA, picric acid.

ing and presence of a number of extra spots show that this epoxypicrate is of questionable purity.

As a rule, the epoxide levels of the 150C and 250C heated oils were too low to permit direct identification. Identification in this case had to be preceded by a preparative step which was done by streaking 1.0–1.5 ml of the reaction solution (containing 40–60 mg of methyl esters) on a TLC plate. This was developed at 5C (in the refrigerator) because, at this temperature, a better separation of *cis*- and *trans*-picrated methyl esters occurs. In addition, development in the refrigerator helps to minimize the tendency of *trans*-methylepoxystearate to decompose in diffuse light. A typical preparative TLC plate showed the characteristics given in Table V.

This preparative step is followed by an identification step in which the eluted TLC streaks in methanol are spotted on an argentated plate and developed at room temperature along with standard fatty acid

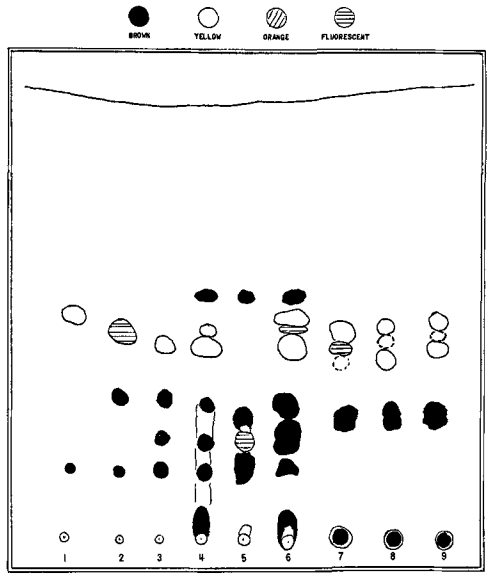

FIG. 4. TLC of picrated epoxides and 80C heated oils: 1) *cis*-methyl epoxystearate; 2) *trans*-methyl epoxystearate; 3) methyl epoxystearate; 4) methyl coronarate; 5) methyl diepoxystearate; 6) mixture of 1–5; 7) olive oil, 80C; 8) cottonseed oil, 80C; soybean oil, 80C.

TABLE VI
Summary of TLC Work on Epoxides in Heated Oils

Sample	Unsaturated		Saturated
	cis-	*trans*-	
80C Heated OO	9X	4X	2X
80C Heated CSO	3X	3X	6X
80C Heated SBO	3X	X	5X
150 Heated OO	6X	5X	3X
150 Heated CSO	5X	3X	2X
150C Heated SBO	4X	3X	2X
250 Heated OO	2X	?	3X
250 Heated CSO	?	X	3X
250 Heated SBO	X	X	3X

picrates. The result of these two steps are summarized in Table VI. It should be emphasized that these results are semiquantitative, at best, because of the number of steps involved and the visual estimation of spot intensities. Another factor that makes quantitation difficult is the instability of the *trans*-band. When this *trans*-fraction is rechromatographed on $AgNO_3$ impregnated plates, a sizeable decomposition spot of lower R_f (0.37 instead of 0.51) is often seen. This is plainly visible in 7, 8, and 9 of Figure 4. It is this decomposition product that seems to be responsible for the fluorescence of picrated *trans*-methyl epoxystearate on standing.

When the combined eluates (bands 3 and 4) of the nine samples were spotted on silica G plates and developed at 5C, the results were identical for all. A large yellow-brown spot at the origin indicated considerable decomposition. This was accompanied by two spots, the smaller one (R_f 0.57), similar to methyl epoxyoleate, and the larger spot with an R_f of 0.52. Neither of these matched the R_f of the main spots of picrated methyl diepoxystearate, hence it is presumed that the R_f 0.52 spot is due to a secondary (yellow) spot of picrated monoepoxides (Fig. 3).

In an effort to determine if diepoxides were present, a large preparative TLC run was made by using a 750 μ thick silica G plate. A 3.5 ml aliquot of picrated 150C heated cottonseed oil solution was applied, and the plate was developed with the standard solvent at 5C.

The three main bands found were eluted with methanol, filtered and evaporated to a minimum volume (about 0.2 ml) under N_2 at room temperature. The solutions of the three bands eluted were then rechromatographed on TLC plates. This once more showed that *trans*-epoxides (band 2) decompose during preparative TLC. Again no diepoxides were detected. Ultraviolet and visible spectra of band 2 did show one anomaly, however. The main peak of its basic solution was at 405 mμ instead of the expected 415–417 mμ. Another difference was encountered in the infrared spectrum of band 2. It had an olefinic C-H stretch peak at 3000 cm^{-1}, suggesting the presence of unsaturated *trans*-epoxides. More TLC work and some GLC data is needed to corroborate the presence of such an entity.

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