The evidence obtained by these countercurrent distributions indicates that safflower oil, like soybean and linseed oils, has essentially a random glyceride distribution pattern. The amounts of the different triglycerides which could be isolated and measured approximate those calculated for a random distribution. In addition, no significant change in the weight curve or composition of the oil was found after interesterification.

Summary

Safflower oil was fractionated in a 200-tube countercurrent distribution apparatus, and the oil was also fractionated after interesterification with C^{14}-labelled palmitic acid. The glyceride composition of the interesterified oil was similar to that of the natural oil. The glycerides were separated on the basis of both unsaturation and chain length of the constituent fatty acids, and the palmitoglycerides had only slightly higher partition coefficients than the oleoglycerides. The amounts of trilinolein, oleodilinolein, and palmitodilinolein found were similar to those calculated for a random distribution. Distribution of a mixture of safflower oil and olive oil showed that no mixing or randomization of triglycerides occurred during countercurrent distribution. It is concluded that fatty acids in safflower triglycerides are distributed in an essentially random pattern.

Acknowledgment

The authors are indebted to L. M. Pultz of the U.S.D.A. Field Crops Research Branch for the safflower samples, to L. H. Mason for the gas chromatographic analyses, to Mrs. M. H. Good and T. L. Mounts for assistance in the laboratory work, and to J. C. Cowan for his interest and encouragement.

REFERENCES

1. American Oil Chemists' Society, "Official and Tentative Methods of Analysis," 2nd ed., cdl-25, rev. to 1956, Chicago, 1946–56.
2. Barker, C., and Hilditch, T. P., J. Oil and Colour Chemists' Assoc., *33*, 6 (1950).
3. Brice, B. A., Swain, M. L., Herb, S. F., Nichols, P. L. Jr., and Riemenschneider, R. W., J. Am. Oil Chemists' Soc., *29*, 279 (1952).
4. Craig, L. C., Anal. Chem., *22*, 1346 (1950).
5. Dutton, H. J., and Cannon, J. A., J. Am. Oil Chemists' Soc., *33*, 46 (1956).
6. Lagawonkar, J. D., Phalnikar, N. D., and Bhide, V. V., J. Univ. Bombay *12A*, pt. 3, 71 (1943).
7. Scholfield, C. R., and Hicks, M. A., J. Am. Oil Chemists' Soc., *34*, 77 (1957).
8. Scholfield, C. R., and Dutton, H. J., Abstracts of Pittsburgh Conference on Analytical Chemistry and Applied Spectroscopy, March 3–7, 1958, p. 30.
9. Vidyarthi, N. L., J. Indian Chem. Soc., *20*, 45 (1943).

[Received May 19, 1958]

Phenolic Antioxidants and the Stability of Perirenal Rat Fat[1]

A. R. JOHNSON, M. W. O'HALLORAN,[2] and F. R. HEWGILL, Commonwealth Antioxidant Research Project, University of Adelaide, South Australia

PREVIOUS investigators have attempted to improve the resistance of extracted animal fats to the onset of rancidity by alteration in the antioxidant composition of the diet. The natural antioxidants, the tocopherols, can be deposited in the fat of the rat (2, 19, 10, 3, 20), swine (4, 5, 33), rabbit (22), turkey (6, 23, 24, 25), and chicken (24, 25, 11). Providing the fatty acid composition is constant, the stability of the extracted fat depends upon the tocopherol content of the diet. Only a very small percentage of the large doses of tocopherol fed however are actually stored in the carcass.

The effect of the ingestion of other antioxidants has been investigated. Ascorbic acid, hydroquinone, α-naphthol, nordihydroguaiaretic acid, lecithin, and tocopherols were fed to rats. Of these only the tocopherols were effective in increasing the stability of the extracted fat (27, 10).

Siedler *et al.* (30) studied the effect on the stability of depot fat extracted from broilers fed a diet supplemented with 6% animal fat stabilized with 0.02% Santoquin (6-ethoxy-2,2,4, -trimethyl-1, 2-dihydroxyquinoline), 0.02% BHT (ditertiary-butyl-p-cresol), or 0.02% DPPD (diphenyl-p-phenylenediamine) for nine weeks. They have shown that the depot fat of birds fed these antioxidants showed little or no increase in stability over the controls.

Pudelkiewiez *et al.* (28) have shown that the depot fat and egg yolk of chickens fed diphenyl-p-phenylenediamine contain this antioxidant in amounts sufficient for estimation.

The use of specified synthetic antioxidants in fats intended for human consumption has been legislatively approved in many countries. The number of compounds so approved is increasing (15). Of those approved, two of the most important are butylated hydroxyanisole (BHA) (16) and butylated hydroxytoluene (BHT) (8). The experiments described in this paper were designed to assess the effect of ingested BHA and BHT upon the stability of extracted perirenal rat fat.

Experimental

Diets. The stock diet (7) consisted of rolled oats 300 g., crushed wheat 300 g., B mixture 300 g., cooked beef mince 80 g., water 200 ml., milk 80 ml., and "Potantol"[3] 0.5 ml. The B mixture contained pollard (shorts) 10 parts, wheat germ 10 parts, lactic casein 7 parts, full cream powdered milk 6 parts, sodium chloride 1.25 parts, and calcium carbonate 0.5 parts.

The stock diet was supplemented by lard to the extent of either 100 or 200 g. per 900 g. of the dry materials of the diet. The total fat content of the supplemented diets was 16.6, or 24.5%, respectively, on a dry weight basis as calculated from standard tables (1).

The freshly rendered lard, guaranteed free of added antioxidants, was purchased at three-month intervals and stored under nitrogen in sealed containers at −15°C. until required. At weekly intervals sufficient lard was removed from nitrogen storage for one week's supply. The antioxidants were added to the

[1] Presented in part at the meeting of the Australian Biochemical Society, Melbourne, June 5–8, 1957.
[2] Present address: Nicholas Institute of Medical and Veterinary Research, Burnham Beeches, Sherbrook, Victoria, Australia.
[3] "Potantol," a commercial vitamin concentrate containing not less than 16,000 I.U. vitamin A and 2,000 I.U. vitamin D per ml.

lard, and the control and experimental lards were dispensed and stored at 0°C. The melted dispensed lard, after homogenization with the liquid components of the diet and the cooked meat, was manually mixed with the remainder of the diet. The supplemented diet was prepared daily except for the week-end and holiday periods when double quantities were given every second day. The animals were supplied with food and water *ad lib.*

Design of the Experiments

BHA.[4] The animals used were part of a large group being employed for a chronic toxicity test for BHA (13). Forty litters of the Norway Hooded strain of rat were selected, each of which contained four males and four females. The effect of sex was considered as one of the variables of the experiment. The male and female weanling rats of the same litter were placed singly in two separate groups of four cages which were then considered as one unit. The diets were randomly allocated so that one brother and sister pair each received one of the following diets of the BHA experiment (Table I).

TABLE I

Fat, BHA, and BHT Content of Diets

Antioxidant	Antioxidant level in lard (%)	Total fat content of diet (% dry weight)	Antioxidant level in diet (% dry weight)
BHA	0.0	16.6	0.0
	0.01	16.6	0.001
	0.004 after heating[a]	16.6	0.0004[a]
	1.0	16.6	0.105
	5.0	16.6	0.523
BHT	0.0	16.6	0.0
	1.0	16.6	0.105
	5.0	16.6	0.523
	0.0	24.5	0.0
	0.5	24.5	0.095
	2.5	24.5	0.474

[a] See results below.

This procedure was repeated four times so that each cage contained four rats. The complete procedure was repeated 10 times, utilizing 320 weanling rats and 80 cages.

The diet containing 0.001% BHA represents an attempt to feed the rat the same level of BHA as man would receive if all his dietary fat, other than that contained as a natural constituent of another food, were stabilized to the extent permitted by the present food laws of several countries (0.01% of the fat). The diet containing 0.105% BHA contains 100 times the permitted dose and is the suggested testing dose for the chronic toxicity of a food additive (17).

During normal culinary practice the lard would be heated. Therefore, prior to mixing into the diet, half of each of the control and experimental lards were heated to and held at 150°C. for 30 min. in Kjeldahl flasks.

BHT.[5] Other work at present in progress in this laboratory suggests that the level of fat in the diet may influence the toxicity and metabolic fate of antioxidants. In the BHT experiment the levels of lard and BHT were varied in such a way that direct comparisons could be made of the effect of a varying fat level with approximately the same antioxidant level, and conversely a constant antioxidant level but variable fat intake.

[4] Tenox BHA, a commercial preparation containing approximately 85% 3-tert-butyl-4-hydroxyanisole and 15% 2-tert-butyl-4-hydroxyanisole.

[5] Tenox BHT, 2:6-ditert-butyl-p-cresol.

One batch of three male weanling litter mates of the Norway Hooded strain of rat was randomly distributed between the 16.6% fat content series of BHT diets and a second between the 24.5% fat content series (Table I). This procedure was repeated eight times, giving eight animals on each diet caged in two groups of four.

In all experiments to be described, the various litters were treated as a unit, *i.e.,* they were sacrificed and fats isolated, stored, and analyzed under the same conditions to ensure valid comparisons.

Solvents

Absolute ethanol was used throughout and purified by refluxing with sodium hydroxide and zinc dust, followed by distillation. Hydroquinone and peroxide were removed from anaesthetic ether by washing with ferrous sulphate and water; the ether was dried over calcium chloride and distilled. In all cases solvents were removed from the fats or other solutions by concentration *in vacuo* under nitrogen.

Modified Method for BHA Estimation

BHA, or lard containing BHA, was dissolved in 15% (v/v) ethanol-benzene and diluted with the same solvent to give a solution containing 50–500 γ/100 ml. At this stage the concentration of lard should not exceed 1%. To 5 ml. of this solution was added 1 ml. of a 0.2% solution of A. R. sodium borate decahydrate in 6% (v/v) methanol-ethanol, followed by 1 ml. of 0.01% (w/v) 2:6-dichloroquinone-chloroimide solution in ethanol. A blank was similarly prepared, using 5 ml. of benzene-alcohol. The color reached a stable maximum after 30 min. when the absorbancy at 620 mμ was determined, using a Hilger "Uvispek" spectrophotometer, and compared with a standard curve.

Effect of Heat on BHA in Lard

At each concentration of BHA, triplicate samples of 100 g. of lard were heated to and held at 150°C. for 30 min. in Kjeldahl flasks connected to condensers arranged for distillation. The condensers were each rinsed with 15% (v/v) ethanol-benzene, and the rinsings were made up to 50 ml. in each case. The BHA content of these solutions was estimated by the modified method.

The BHA content of all the lard samples, before and after heating, was estimated by the modified method and by Mahon and Chapman's method (21) in the case of the lowest concentration.

Extraction of the Fat

The rats were sacrificed by a blow on the nape of the neck and exsanguinated. The perirenal fat was isolated and homogenized for 90 seconds in ethanol-ether (3:1) in the presence of anhydrous sodium sulphate. After filtration through Celite the solvent was removed. The fat was stored at −15°C. until analyzed. In the majority of cases the analysis was carried out on the following day.

Estimation of Stability

It has been established (12, 19) that the Warburg respirometer method is eminently suitable for the determination of fat stability. A conventional Warburg apparatus was modified to operate at 100°C. Duplicate samples of 0.1 ml. of the fat were placed in the center well of the cups (14-ml. capacity), and the cups were flushed with oxygen for 15 min. The oxygen uptake was measured in the usual manner after

TABLE II
Effect of Ingested BHA on the Stability of Perirenal Rat Fat. Induction Period in Minutes at 100°C.

BHA content of diet, % dry weight	Male					Female				
	0.0	0.001	0.0004	0.105	0.523	0.0	0.001	0.0004	0.105	0.523
Unheated lard	21	40	....	148	462 } [a] 450 }	300	347	....	600	438
	35 } [a] 36 }	164	....	192	301	165	230	....	343	504
	100	85	....	220	210	180	118	....	....	263
	121	111	....	163	207	39	101	....	302	311
	94	70	....	185	364	200	61	....	385	537
Mean value	74	95	....	182	309	177	171	....	408	411
Heated lard	33	....	42	239	184	29	....	163	222	175
	132	....	194	157	259	68	....	119	63	260
	51	....	50	155	302	68	....	107	228 } [a] 240 }	869
	51	....	56	90	180	145	....	139	258	258
	9	....	10	138	142	84	....	370	350	384
Mean value	55	....	70	156	213	79	....	180	224	389

Each row represents one set of litter mates.
[a] Perirenal fat from these rats divided into two unequal portions and treated separately.

10 min. of equilibration in the light mineral oil bath with the taps open. The stability was measured by the induction period in minutes, which was the time required for the onset of the rapid uptake of oxygen. The observation of Lundberg *et al.* (19) that the onset of the rapid uptake of oxygen for rat fat was sufficiently sharp to define the induction period within the time interval between readings, *i.e.* 10 minutes, was confirmed.

Estimation of the Iodine Value

Estimation of the iodine value was by the Wijs (30-min.) method).

Isolation of the Stabilizing Principles

Procedure I for BHA and BHT. The extracted fat was dissolved in ethanol-ether (5:1) and allowed to crystallize at −15°C. for 3 days. The fat was filtered off, avoiding any appreciable temperature rise. The solvent was removed from the filtrate, and the residue steam was distilled. The distillate was extracted four times with redistilled A.R. benzene, the combined extracts were dried (sodium sulphate), and the solvent was removed.

Procedure II for BHA. The fat was dissolved in hexane, and the solution was extracted 3 times with 72% (v/v) aqueous ethanol, a selective solvent for BHA (21). The ethanol solution was desiccated with anhydrous sodium sulphate, and the ethanol was removed. The residue was dissolved in hexane and successively chromatographed on magnesium trisilicate and alumina columns. The BHA was removed from the alumina columns by 15% (v/v) ethanol-benzene (14).

Results

BHA

Estimation. Pure solutions of BHA and solutions of BHA in lard can be estimated by the simplified procedure described under Experimental, provided the concentration of lard does not exceed 1% in the final solution to which the reagents are added. Beer's law is obeyed in the range 0–800 γ/100 ml. When used with pure solutions of BHA, the extinction coefficient $E_{1\%}^{1cm.} = 790 \pm 3.12$ [6] compared favorably with the value of $E_{1\%}^{1cm.} = 810.1 \pm 20.32$ [6] obtained by carrying out determinations in 72% aqueous ethanol, as in the Mahon and Chapman (21) procedure.

Estimation of BHA in lard before and after heating showed a negligible loss in the two highest concentrations (1% and 5%); however in the lowest concentration (0.01%) approximately 60% loss occurred. This was confirmed, using the method of Mahon and Chapman (21). Losses due to volatilization of BHA were negligible in all cases.

[6] Standard error of the mean of duplicate determinations carried out in the range 200–800γ/100 ml. inclusive.

Fat Stability

The effect of ingested BHA on the stability of the extracted perirenal rat fat of 4–5-month-old rats is shown in Table II.

In three instances the isolated perirenal fat was divided into two unequal portions. These were extracted, and their stability was estimated as separate units. The agreement between the results of the two portions of the same fat [see (a), Table II] indicated that neither the quantity of fat nor the method of extraction and estimation of stability introduced significant errors.

A statistical analysis of the figures showed that the means for the treatments containing 0.105 and 0.523% BHA in the diet were significantly higher ($p << 0.001$) than the mean of the control. The mean for the diet containing 0.001% was higher than the control, but the increase is not quite significant ($t = 1.92$ on 47 degrees of freedom, 5% level of significance $t = 2.02$).

The stability of the fat isolated from the female rats was significantly greater ($p < 0.05$) than that isolated from the males. There was also a significant increase ($p < 0.01$) in the stability of the fat isolated from those rats fed unheated lard over those fed heated lard.

The stabilities were estimated to be in the ratios

% BHA	Control	0.001%	0.105%	0.523%
Ratio of stability	1	1.37	2.97	4.20

Since an analysis of variance of the logarithms of the induction periods showed no interactions, these ratios are the same, to within limits of experimental error, for both sexes and for heated and unheated. Similarly the absence of interactions enables a figure to be quoted for the over-all percentages for the sex difference and the effect of heating. Females have an 87% higher stability than males in both heated and unheated groups, and heating reduces stability by 31% in both sexes. These percentages are the same for each level of BHA.

Iodine Value

Ingested BHA caused significant changes in the composition of the extracted perirenal rat fat in

both male and female animals, whose diet contained 0.001% BHA in unheated lard, and in those whose diet contained 0.523% BHA in heated lard (Table III). The former caused a depression of the iodine value ($p<0.001$) while the latter caused an elevation of the iodine value ($p<0.02$) over the appropriate controls.

TABLE III
Effect of Ingested BHA on the Iodine Value of Perirenal Rat Fat

	Mean iodine value of respective treatments				
BHA content of diet % dry weight	0	0.001	0.0004	0.105	0.523
Unheated lard	72.7	68.3	....	72.1	72.7
Heated lard	71.1	....	72.3	71.8	73.7

Least significant difference at 5% level (L.S.D.5%) between means is 2.1.

BHT

Table IV shows the effect of dietary BHT upon the stability of perirenal fat of 6-month-old male rats. All concentrations of BHT caused a significant increase ($p<0.001$) in the stability of the fat on both fat regimes.

An analysis of variance of the iodine values of the perirenal fat from those animals within the BHT experiment showed that those animals whose diet was supplemented with 20% lard had a significantly lower iodine value ($p<0.05$) than those whose diet was supplemented with 10% lard (Table V). The analysis also showed that there was an indication of a significant trend toward a reduction in the iodine value on inclusion of BHT in the diet. Upon taking out the regression coefficient, this trend was shown to be significant ($p<0.05$). The increase in the amount of lard in the diets of both control and experimental rats caused the iodine value of the perirenal fat to fall to a level more closely approximating that of lard (I.V. 56). As the stability of a fat depends upon the type and degree of unsaturation, one must compensate for the increased stability when comparing the effects of diets containing a higher fat level but the same quantity of BHT.

For this purpose use was made of the antioxidant index, which is defined as the ratio of the induction period of the experimental to that of the control fat. The antioxidant indices for the two fat regimes were not statistically different at the 5% level. This was confirmed by an analysis of variance of the induction periods when it was shown that there was no interaction between the dietary levels of fat and BHT.

TABLE IV
Effect of Ingested BHT on the Stability of Male Perirenal Rat Fat

Fat content of diet, % dry weight	Induction period in minutes at 100°C.					
	16.6%			24.5%		
BHT concentration in diet, %, dry weight	0.0	0.1045	0.523	0.0	0.095	0.474
	252	372	633	298	471	968
	96	223	400	132	475	1183
	100	216	732	119	357	765
	102	385	485	136	490	655
	90	270	406	130	380	870
	114	307	705	133	453	875
	121	417	750	159	480	831
	189	425	655	201	550	1072
Means	133	327	585	163	457	904

Within the two fat regimes each row represents litter mates.

TABLE V
Effect of Ingested BHT on the Iodine Value of Perirenal Rat Fat

Approximate BHT content of diet, % dry weight	Mean iodine value for respective treatments			
	0	0.1	0.5	Lard means
10% lard supplemented to diet	74.5	74.1	72.5	73.7
20% lard supplemented to diet	71.3	70.6	70.3	70.7
Treatment means	72.9	72.3	71.4	....

L.S.D. 5% between treatment means is 1.2.

A 10% coconut oil supplement to the stock diet was also effective as a carrier for BHT (0.105% dry weight of diet) (Table VI). In this case 24-month-old Wistar Albino rats were used, and the perirenal fat of surviving litter mates of a chronic toxicity test were compared for stability at 80°C. Because of the different testing conditions the results of this experiment cannot be compared directly with those reported above. Nevertheless they confirm the stabilization of extracted rat fat by ingested BHT ($p<0.05$), also the difference between the stability of fat from male and female rats ($p<0.01$).

TABLE VI
Effect of Ingested BHT in Coconut Oil on the Stability of Perirenal Rat Fat

	Induction period in minutes at 80°C.	
	Control	0.1% diet BHT (dry weight)
Male	197	415
	82	157
	80	660
	203	430
Means	140	415
Female	1523	4006
	425	703
Means	972	2354

Litter mate on same row.

Isolation of Stabilizing Principles

Attempts were made to isolate the materials responsible for the increased stability. The steam distillation method (procedure I, Experimental) was applied to 20-g. samples of pooled control and experimental fats from the main BHA and BHT experiments. In both cases material was isolated from the experimental fats, which gave the characteristic color reaction for the appropriate antioxidant. Use was made of these color reactions, *i.e.*, the 2:6-dichloroquinone chloroimide reaction for BHA (9, 14) and a modification of the phosphomolybdic acid method for BHT (32), to estimate the amount of material present on the assumption that the material estimated was the antioxidant in question. There was 2 γ "BHA"/gram experimental BHA fat and 10 γ "BHT"/gram experimental BHT fat. No "BHA" was present in the control fat, but a small amount of material reacting as "BHT" was present in the control fat probably because of the unspecific nature of the phosphomolybdic acid method.

Similar amounts of "BHA" were shown to be present in the BHA experimental fat when the ethanol and chromatographic method (procedure II, Experimental) was used.

When the substances which had been isolated from the control and experimental fats were added to

portions of the original control fat, its stability was increased in the ratio:

Additions to control fat	0	Control extract	"BHA" extract	"BHT" extract
Ratio of stabilities	1	4.8	8.5	9.5

In all cases the extracts represented a 10-fold concentration of the substances present in the original fats. The amounts of "BHA" and "BHT" isolated were insufficient for positive identification.

Discussion

We have established that the ingestion by rats of BHA and BHT in amounts 100 and 500 times the level generally permitted in fats for human consumption causes an increase in the stability of the extracted perirenal fat. The lack of a significant increase of stability of those animals fed BHA at the normal level of usage is in agreement with the results of Siedler *et al.* (30).

The fact that materials can be isolated which possess antioxidant activity and give the characteristic color reaction for the appropriate antioxidant strongly suggests that these antioxidants or some close derivative are deposited in the depot fat. The amounts of "BHA" and "BHT" isolated from the depot fat were only a very small fraction of the amounts ingested. This finding is in accord with the result of other workers (see introduction) on the natural antioxidants, the tocopherols.

Whether these antioxidants or some close derivative are directly responsible for the increased stability cannot be ascertained at this stage. A possibility that cannot be excluded is that the synthetic antioxidants may stabilize the natural antioxidants and thus lead to their accumulation in the depot fat.

The changes of the iodine value of the extracted fat upon the ingestion of BHA and BHT must reflect changes in the composition of that fat. However, as Longenecker (18) has pointed out, the iodine value is a relatively crude index of composition of a fat. In the present authors' opinion the changes of the iodine value are not sufficiently large and, in the case of BHA, are in the wrong direction to explain the increases in stability. It must be remembered however that a relatively large change in the small content of the polyunsaturated acids, one of the chief factors responsible for the onset of rancidity, may not be reflected in changes in the iodine value. An investigation into the possible effects of antioxidants on the composition of depot fat, using more refined techniques, is at present being undertaken in this laboratory.

The stability of the female rat fat was significantly higher than the male even in those animals whose diet contained no added antioxidants. The explanation of the difference is probably a combination of several factors. The iodine values of the male and female fats were not significantly different. However Longenecker (18) compared the composition and iodine value of the depot fat of male and female rats of the same age and on the same diet and showed that there was a considerable sex difference in the fatty acid composition which was not reflected in the iodine values.

Quaife and Dju (29) investigated the tocopherol content of a male and a female subject and found that there was a greater tocopherol content in the female than in the male. The validity of this observation is open to question because of the unknown age and dietary habits of the two subjects. A more significant point was their finding of appreciable amounts of *gamma* and *delta* tocopherols in all fat from the female and their presence only in the subcutaneous fat of the male. *Delta* tocopherol has been shown to possess the greatest antioxidant activity of the tocopherol isomers (20).

A further possibility which might explain the increased stability of the female fat is related to the hormonal differences between the sexes. Meyer and McShan (26) and Schuler and Meir (31) have shown that the oestrogens as distinct from the androgens can act as antioxidants while Hood *et al.* (11) showed that the fat from chickens given oestrogen implants was more stable than control fat.

The fact that ingested antioxidants currently used in fats for human consumption can materially effect the stability of isolated rat fat suggests that this might occur in humans ingesting stabilized fats and emphasizes the need for further knowledge on the metabolic role and fate of these antioxidants.

Summary

1. Ingested BHA and BHT, in amounts 100 and 500 times the normal level of usage in fats for human consumption, increase the stability of extracted perirenal rat fat. At the normal level of usage of BHA an increase in stability occurred which was not quite significant. The increases are related to the amount of antioxidant in the diet.

2. Pre-heating of lard, and lard stabilized with BHA, causes a 31% reduction of the stability of both male and female perirenal rat fat on the same level of BHA. The reduction in stability cannot be ascribed to the heat destruction of BHA at the two highest concentrations used.

3. Irrespective of the presence or absence of BHA, female rat fat is 87% more stable than the male in both heated and unheated groups on the same level of BHA.

4. The presence in the diet of varying amounts of lard at the same level of BHT did not influence the increases in stability caused by BHT.

5. The supplementation of the diet with 10% of a 1% solution of BHT in coconut oil increased the stability of male and female perirenal rat fat.

6. The iodine value of male and female rat fat was lowered by a diet supplemented with 10% unheated lard containing 0.01% BHA and was raised by a diet supplemented with 10% heated lard containing 0.5% BHA.

7. Reductions in the iodine value of rat fat are produced in those animals whose diet contained 0.1% and 0.5% BHT.

8. Attempts to isolate the stabilizing principles proved only partially successful. Materials possessing antioxidant activity and giving the usual color reactions for the appropriate antioxidant were isolated in amounts insufficient for characterization.

9. Possible mechanisms to explain the observed differences are discussed.

Acknowledgments

The authors wish to acknowledge the generous gifts of Tenox BHA and BHT, supplied by Eastman Chemical Products Inc., and to thank A. T. James

of the Division of Mathematical Statistics, C.S.I.R.O. for the statistical analyses, Miss E. Wadlow for technical assistance, and A. McNeil for his care of the animals. They would also like to acknowledge the constructive criticisms given by J. R. Vickery and J. Shipton during the preparation of this paper.

REFERENCES

1. Albritton, E. C., "Standard Values in Nutrition and Metabolism" (1954).
2. Barnes, R. H., Lundberg, W. O., Hanson, H. T., and Burr, G. O., J. Biol. Chem., *149*, 313 (1943).
3. Burr, G. O., Lundberg, W. O., and Chipault, J. R., Oil and Soap, *23*, 382 (1946).
4. Carpenter, L. E., and Lundberg, W. O., Ann. N. Y. Acad. Sci., *52*, 269 (1949).
5. Chipault, J. R., Lundberg, W. O., and Burr, G. O., Arch. Biochem., *8*, 321 (1945).
6. Criddle, J. E., and Morgan, A. F., Proc. Soc. Expt. Biol. Med., *78*, 41 (1951).
7. C.S.I.R.O., Biochemistry and General Nutrition Division, Adelaide, personal communication.
8. Dugan, L. R. Jr., Marx, Lotte, Weir, C. E., and Kraybill, H. R., Am. Meat Institute Foundation, Bull. 18 (June 1954).
9. Gibbs, J., J. Biol. Chem., *72*, 649 (1927).
10. Hanson, H. T., Barnes, R. H., Lundberg, W. O., and Burr, G. O., J. Biol. Chem., *156*, 673 (1944).
11. Hood, M. P., Wheeler, R. S., and McGlamery, J. B., Poultry Sci., *29*, 824 (1950).
12. Johnston, W. R., and Frey, C. N., Ind. Eng. Chem., Anal. Ed., *13*, 479 (1941).
13. Johnson, A. R., and O'Halloran, M. W., in preparation.
14. Johnson, A. R., O'Halloran, M. W., and Hewgill, F. R., in preparation.
15. J. Agr. Food Chem., *4*, 667 (1956).
16. Kraybill, H. R., Beadle, B. W., Vibrans, F. C., Swartz, V. N., Wilder, O. H. M., and Rezabek, Helen, Am. Meat Institute Foundation, Bull. 2 (April 1948).
17. Lehman, A. J., Fitzhugh, O. G., Nelson, A. A., and Woodard, G., Adv. Food Res., *3*, 197 (1951).
18. Longenecker, H. E., J. Biol. Chem., *128*, 645 (1939).
19. Lundberg, W. O., Barnes, R. H., Clausen, Marion, and Burr, G. O., J. Biol. Chem., *153*, 265 (1944).
20. Lundberg, W. O., Barnes, R. H., Clausen, Marion, Larson, Norma, and Burr, G. O., J. Biol. Chem., *168*, 379 (1947).
21. Mahon, J. H., and Chapman, R. A., Anal. Chem., *23*, 1,116 (1951).
22. Major, R., and Watts, B. M., J. Nutrition, *35*, 103 (1948).
23. Mecchi, E. P., Pool, M. F., and Klose, A. A., Poultry Sci., *32*, 915 (1953).
24. Mecchi, E. P., Pool, M. F., Behman, G. A., Hamachi, M., and Klose, A. A., Poultry Sci., *35*, 1238 (1956).
25. Mecchi, E. P., Pool, M. F., Nonaka, N., Klose, A. A., Marsden, S. J., and Lillie, R. J., Poultry Sci., *35*, 1,246 (1956).
26. Meyer, R. F., and McShan, W. H., Biological Antioxidants, Trans. 3rd Conf., Josiah Macy Jr. Foundation, 115 (1948).
27. Overman, A. J., J. Biol. Chem., *142*, 441 (1942).
28. Pudelkiewicz, W., Potter, L. M., Matterson, L. D., and Singsen, E. P., Poultry Sci., *35*, 959 (1956).
29. Quaife, M. L., and Dju, M. Y., J. Biol. Chem., *180*, 263 (1949).
30. Siedler, A. J., Moline, Sheldon, Schweigert, B. S., and Riemenschneider, R. W., Poultry Sci., *36*, 449 (1957).
31. Schuler, W., and Meiser, R., Arch. Expt. Pathol. Pharmakol., *228*, 474 (1956).
32. Shell Chemical Corporation, Technical Bulletin, Sci., 55-43, 4 (1955).
33. Watts, B. M., Cunha, T. J., and Major, R., Oil and Soap, *23*, 254 (1946).

[Received December 16, 1957]

Fractionation of Sesame and Safflower Oil Fatty Acids with Urea

T. N. MEHTA and S. B. DABHADE, Laxminarayan Institute of Technology, Nagpur University, Nagpur, Bombay State

THE FORMATION of urea complexes with straight-chain organic compounds was first discovered by Bengen (3). The stability of these complexes varies greatly according to the type of compound complexed. This phenomenon makes it possible to achieve separation of certain organic compounds by varying the temperature, urea concentration, and solvent concentration.

Straight-chain compounds form much more stable adducts with urea than do cyclic or branched-chain compounds. Bengen (3), Rudloff (11), and Mehta and Sharma (9) were able to obtain fairly satisfactory separations of straight-chain compounds from cyclic and branched-chain compounds.

Recent investigations (1, 8, 10, 12, 13, 14) have shown that it is possible to separate mixtures of fatty acids or methyl esters of fatty acids on the basis of their degree of unsaturation. These studies have shown that the stabilities of urea adducts decrease as their degree of unsaturation increases.

This investigation was conducted to study the fractionation and separation of fatty acids according to their degree of unsaturation. Both the methyl esters and mixed fatty acids of sesame and safflower oils have been used. A comparison is made between two different methods of fractionation. In one method increasing increments of urea are added to the fatty acid-solvent systems to obtain increasingly unstable urea adducts. In the other method the mixed fatty acids are completely converted into urea adducts and eluted with varying amounts of solvent. By this method the most unstable components are eluted first while additional increments of solvent allow the more stable components to be progressively eluted.

Experimental

Preparation of Methyl Esters. Methyl esters of sesame (I.V. −106.4, S.V. −188.6) and safflower (I.V. −146.0, S.V. −190.4) oils were prepared by interesterification of the oils and anhydrous methanol by the method of Bradley and Johnston (4), using potassium hydroxide as the catalyst. The esterified product was then washed with acidulated ice-cold distilled water and extracted with ethyl ether. The extract was washed free of mineral acid and dried over anhydrous sodium sulfate, and the ether was removed under vacuum.

Preparation of Mixed Fatty Acids. The mixed fatty acids of sesame and safflower oils were prepared by the method of Hilditch (7).

Fractionation by the Urea Adduct Elution Method. The general procedure followed for this method was to dissolve the methyl esters or fatty acids in a mixture of powdered urea and 95% ethanol. The mixture was heated and kept over-night at room temperature. The adduct obtained was filtered through a sintered glass funnel by suction. An additional amount of ethanol was added to the adduct, and the filtration procedure was repeated. This elution technique was repeated a number of times. Each of the filtrates thereby obtained was concentrated by removing the ethanol under reduced pressure. The residue was treated with acidulated distilled water, washed free of mineral acids, and extracted with ether. The ether extracts were dried over anhydrous sodium sulfate and evaporated to remove the ether. Each of these fractions was analyzed for iodine and saponification values.

The fatty acid composition of each fraction was calculated from the iodine and saponification values by assuming the fractions to be binary mixtures. This assumption has also been made by other investigators (2, 5, 6, 7) in calculating the fatty acid composition of various fats. The mean molecular weight of the