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Microdetermination of Polyenoic Fatty Acids and Total Fatty Acids in Plasma and Tissue

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A method for the isolation of fatty acids from 2 g plasma or tissue-homogenate prior to the determination of polyenoic acids is described. Total fatty acids are determined by titration. The polyenoic acids are determined by the method of Herb and Riemenschneider, the amount of fatty acids isomerized being 2 – 6 μequiv. This method gives highly reproducible results. The saponification step of the isolation procedure was investigated with respect to changes in composition and structure of the fatty acids during saponification. It was found that the degree of conjugation of double bonds was low, and that the proportions of the different polyenoic acids were not influenced by saponification.

Since Herb and Riemenschneider [1] published the micro-isomerization method for the determination of polyenoic fatty acids, their method has been used by several investigators [2–14] on extracts from biological materials and has proved to be a useful tool in lipid research.

The determination of polyenoic fatty acids in biological material necessitates the isolation of the fatty acids prior to their isomerization. Numerous methods for this purpose have been published, but only few authors [10–12] have given information with regard to changes in composition and structure of the polyenoic fatty acids during isolation of fatty acid mixtures from biological material. An important step is the saponification of the lipid extract since it is well known that conjugation of double bonds takes place when polyenoic fatty acids are treated with alkali. In order to avoid this, some workers [13,15] omit saponification and isomerize isolated esters. Nevertheless, for more accurate determinations on most biological materials, saponification seems to be necessary, since both Front and Daubert [16] and Holman and Hayes [15] have shown that cholesteryl esters are isomerized much more slowly than the free acids.

The present paper reports the result of an investigation on the extraction and saponification of lipids from 2 g plasma or tissue homogenate (*ca.* 1:10 in water), the polyenoic fatty acids being determined by the method of Herb

and Riemenschneider [1]. Simple and accurate methods for the isolation of fatty acids from biological material and for the determination of total fatty acids on the same sample as used for the determination of polyenoic fatty acids are described.

REAGENTS AND METHODS

Reagents

1. *Extraction and isolation of unsaponifiable and saponifiable fractions.*

Ethanol, aldehyde-free. 1.5 l 96 % ethanol is boiled for 12 h with 30 g KOH and 30 g Zn-powder and distilled.

Ethyl ether, peroxide-free. 1 l ethyl ether is stored for 2 days with 50 g KOH. The peroxide-free ether is obtained by decantation.

4 N ethanolic KOH (Merck p.a.). Should be prepared immediately before use.

Petroleum ether, b.p. 30—45°C, is prepared by fractional distillation of commercial petroleum ether, b.p. 50°C.

2. *Determination of polyenoic fatty acids.*

Isomerizing reagent. 21 % KOH in ethylene glycol (w/w), Merck p.a., is prepared as described by Pikaar and Nijhof [12]. The reagent is adjusted to 21.0 ± 0.1 % (after titration of a sample with 0.1 N HCl) with ethylene glycol.

Optically pure petroleum ether. 500 ml petroleum ether, b.p. 60—70°C, is stirred with 100 ml fuming H_2SO_4 for 30 min; the phases are allowed to separate and the petroleum ether is washed with water, 5 % NaOH and water. The whole procedure is repeated. The petroleum ether is dried over anhydrous Na_2SO_4 and distilled. Petroleum ether prepared in this way has a smooth, flat absorption curve (with water as the blank), the transmission being greater than 85 % in the region 220—400 mμ.

Lauryl alcohol, BDH.

Methanol, Merck, pure acetone-free.

3. *Titration of total fatty acids.*

0.025 N NaOH in water.

0.1 % thymol blue in 96 % ethanol.

Methods

1. *Preparation and extraction of plasma and tissue.*

1.1. Plasma. A 2 ml syringe provided with a blunt cannula (length 120 mm, inner diameter 1.5 mm) is filled with plasma and weighed. The plasma is added dropwise, with agitation, to 60 ml ethanol-ether 3:1 in a 100 ml volumetric flask (B 10 ground joint). The syringe is weighed again and the amount of plasma delivered is calculated. The extract is boiled for ½ min., cooled to 20°C and made up to volume with ethanol-ether 3:1. After mixing, the solution is filtered rapidly through a fat-free filter (Munktell No. 3, 9 cm, extracted with ether) and 40 ml extract is pipetted into a 100 ml flask provided with B 29 ground joint. Pumice is added and *ca.* 30 ml solvent is evaporated at 40°C in vacuum. Again 40 ml extract is transferred to the flask, the solvent is evaporated in vacuum until about 10 g (12 ml) is left, and the extract is ready for saponification (see 2).

1.2. Tissue. At least 0.5 g wet tissue is weighed into a beaker, 7—10 volumes of water is added and the beaker is weighed again. The tissue is homogenized for a few minutes with an Ultraturrax disintegrator, TP 18/2, and 2 g homogenate is treated as described for plasma. Homogenization in a Potter Elvehjem homogenizer was used in some experiments on liver.

2. *Saponification and extraction of the unsaponifiable and saponifiable fractions.*

To 12 ml extract prepared as described under 1, 1 ml H_2O and 4 ml 4 N ethanolic KOH is added. The mixture is boiled under reflux for 60 min. and after addition of 15 ml H_2O the flask is cooled to room temperature. The mixture, which should now contain 50 % ethanol, is extracted in the saponification flask with 4 × 10 ml petroleum ether, b.p. 30—45°C. After each extraction the extract is transferred by light suction to a 50 ml volumetric flask as shown in Fig. 1. Aliquots of this extract may be used for determination of total cholesterol. Now 4.5 ml 4 N HCl and 4.5 ml ethanol is added and

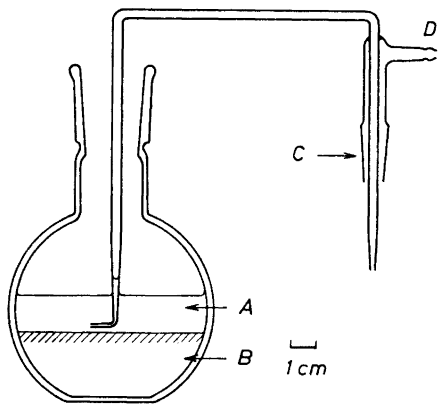

Fig. 1. Set-up for the extraction of unsaponifiable matter and fatty acids after saponification. A: petroleum ether, B: saponified mixture (50 % ethanol), C: B 10 ground joint connected to volumetric flask. By light suction at D the petroleum ether is transferred to the volumetric flask.

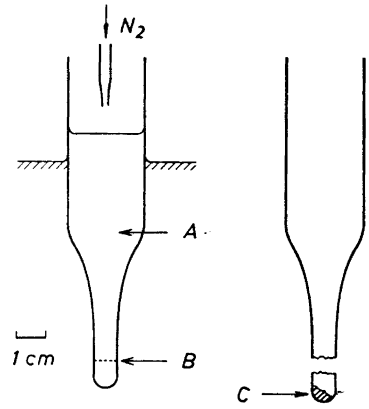

Fig. 2. Special tube used for the transfer of fatty acids from a petroleum ether aliquot to the isomerization tube. Left: tube with aliquot of fatty acid extract (A), B: scratch. Right: tube with bottom broken off, C: vial containing the fatty acids from the petroleum ether aliquot.

the fatty acids extracted as described for the unsaponifiable fraction. The volumetric flask containing the extract is made up to 50 ml with petroleum ether, b.p. 30—45°C, and aliquots of the extract are used for the determination of polyenoic fatty acids and total fatty acids.

3. *Determination of total fatty acids.*

For each determination a 5 ml aliquot of the fatty acid extract is transferred to a small test tube with flat bottom (70 × 14 mm) and the solvent is evaporated in vacuum. The fatty acids adhering to the sides of the tube are rinsed down with 0.5 ml solvent, which is evaporated. Now 1 ml ethanol containing 1 drop 0.1 % thymol blue per ml and 2 small stainless magnetic balls (diameter = 2—4 mm) are added, and the fatty acids are titrated with 0.025 N NaOH from the microburette described by Holm-Jensen [17] with magnetic stirring. CO_2-free N_2 is blown over the sample during titration. For each set of determinations 3 blanks (1 ml ethanol with thymol blue) and 3 standards (5 ml of a 0.36 mN solution of stearic acid in petroleum ether, treated as 5 ml fatty acid extract) are titrated.

4. *Determination of polyenoic fatty acids.*

4.1. Analysis for polyenoic fatty acids with non-conjugated double bonds is performed in duplicate on 20 ml fatty acid extract. 10 ml extract is placed in a special tube shown on Fig. 2. Two such tubes are easily made from an ordinary test tube (180 × 24 mm) from which the bottom has been removed. Before use, the special tubes are ringed with a scratch *ca.* 10 mm from the bottom. The tube containing the 10 ml aliquot is placed in a water bath at *ca.* 40°C, the solvent is evaporated under nitrogen, and during the evaporation the inner wall of the tube is rinsed 3—4 times with petroleum ether. By this procedure the fatty acids are concentrated in the bottom of the tube which can now be broken off by touching the scratch with a hot glass rod. The resulting small vial containing the fatty acids is transferred to an isomerization tube containing reagent preheated for 10 min. at 180°C and the isomerization performed at the conditions employed by Herb and Riemenschneider [1] (5 g 21 % KOH in ethylene glycol, 15 min., 180 ± 0.5°C under N_2). If the samples are not isomerized immediately after evaporation of the solvent, the dried fatty acids will easily oxidize. Covering the sample with a few drops of lauryl alcohol will protect against oxidation for at least 2 h.

After isomerization the sample is mixed with 2 ml methanol and the mixture is transferred by 3—4 washings with methanol to a 25 ml or 50 ml volumetric flask by a method similar to that shown in Fig. 1. Extinctions are determined with a Beckman DR at 400—230 mμ or with a Beckman DU at 375, 346, 315, 268, and 233 mμ with slit widths 0.2, 0.2, 0.3, 0.4, and 0.8 mm, respectively. The hydrogen lamp is used as light source at all wavelengths mentioned.

4.2. The spectra of the non-isomerized fatty acids are obtained from the residue of the fatty acid extract after aliquots have been drawn for determinations of polyenoic fatty acids and total fatty acids. The solvent is evaporated under N_2 and 10 ml optically pure petroleum ether is added. The extinctions are then determined with a Beckman as described for isomerized samples.

4.3. Calculations. Several authors have published spectral constants ($K = E^{1g/l}_{1cm}$) for pure samples of linoleic acid [1,18], linolenic acid [1,18], arachidonic acid [1,19], eicosapentaenoic acid [1,18-21,23], docosapentaenoic acid [1,20,21] and docosahexaenoic acid [18,20-23] isomerized by the method of Herb and Riemenschneider [1]. A critical examination of the purity criterions published for the above mentioned acids seems to justify the choice of the constants published by Herb and Riemenschneider [1] for linoleic, linolenic and arachidonic acids and those published by Holman [22] for eicosapentaenoic and docosahexaenoic acids. These constants are collected in Table 1 as $E^{1\,g/l}_{1\,cm}$ and $E^{1\,mequiv./l}_{1\,cm}$ ($= E^{1\,g/l}_{1\,cm} \times$ mol.wt. $\times 10^{-3}$) and are used in this investigation. The author has determined the spectral constant for linoleic acid at 233 mμ (K^{diene}_{233}) on a sample (Hormel) containing *ca.* 1.2 % triene and 0.5 % conjugated diene. The amount of diene (linoleic acid) in the sample was calculated as: % diene = 100 − % triene − % conjugated diene. After isomerization of samples from 0.1 mg to 12 mg, K^{diene}_{233} was calculated by the following equations:

$$k'_2 = K^{diene}_{233} \cdot \frac{\%\ diene}{100} + K^{triene}_{233} \cdot \frac{\%\ triene}{100}$$

$$K^{diene}_{233} = \frac{100 \cdot k'_2 - K^{triene}_{233} \cdot \%\ triene}{\%\ diene}$$

where k_2' is the increase in specific extinction coefficient at 233 mμ due to isomerization. K^{triene}_{233} was taken from Table 1 because this value was confirmed in a similar experi-

Table 1. Spectral constants for polyenoic fatty acids isomerized according to Herb and Riemenschneider [1].

Acid	Mol.wt.	K_{233}		K_{268}		K_{315}		K_{346}		K_{375}	
		$E^{1\,g/l}_{1\,cm}$	$E^{1\,mequiv./l}_{1\,cm}$	$E^{1\,g/l}_{1\,cm}$	$E^{1\,mequiv./l}_{1\,cm}$	$E^{1\,g/l}_{1\,cm}$	$E^{1\,mequiv./l}_{1\,cm}$	$E^{1\,g/l}_{1\,cm}$	$E^{1\,mequiv./l}_{1\,cm}$	$E^{1\,g/l}_{1\,cm}$	$E^{1\,mequiv./l}_{1\,cm}$
Linoleic *	280.44	91.6	25.69								
Linolenic *	278.42	47.5	13.22	90.5	25.20						
Arachidonic*	304.46	39.7	12.09	48.2	14.67	60.6	18.45				
Eicosapentaenoic **	302.44	37.0	11.19	32.9	9.95	71.1	21.50	74.1	22.41	4.37	1.322
Docosahexaenoic **	328.48	43.9	14.42	54.0	17.74	32.8	10.77	29.4	9.66	28.6	9.39

* Herb and Riemenschneider [1]
** Holman [22]

Table 2. Isolation of fatty acids from tissue and plasma. The samples were extracted with 60 ml ethanol:ether, the extract saponified and the fatty acids extracted with petroleum ether after acidification of the saponification mixture.

Sample	Weight of sample extracted g	Total fatty acids mequiv./100 g plasma or tissue
Human plasma 1.	1.046	1.01
	1.232	1.03
	1.681	1.04
	2.004	1.01
Human plasma 1. diluted 1:3 with 0.9 % NaCl	5.042	1.04
	5.067	1.06
Human plasma 2.	2.076	0.84
	2.181	0.80
Human plasma 3.	2.131	0.71
	2.145	0.69
Rat heart homogenate 1:10 in water	1.968	6.87
	2.111	6.73
	4.849	6.54
	5.146	6.43
Calf liver homogenate 1:10 in water	0.922	11.14
	0.957	11.10
	2.013	10.81
	2.062	10.88
	4.878	10.81
	5.101	10.34

ment with linolenic acid in the authors laboratory. In this way K^{diene}_{233} was found to be 91.2 ± 0.3 which is in close agreement with the value found by Herb and Riemenschneider [1].

By the following set of equations the content of the different types of polyenoic fatty acids is calculated as weight % of total fatty acids. These equations are used when the weight of the fatty acids isomerized is known, and are based on the $E^{1\,g/l}_{1\,cm}$ -values in Table 1. k'_2, k'_3, k'_4, k'_5, and k'_6 denote the increase induced by isomerization in the specific extinction coefficients of the sample ($E^{1\,g/l}_{1\,cm}$) at 233, 268, 315, 346, and 375 mμ, respectively. The k'-value is obtained as the difference between the specific extinction coefficient after (k'_{233}, k'_{268}, k'_{315}, k'_{346}, and k'_{375}) and before (k_{233}, k_{268}, k_{315}, k_{346}, and k_{375}) isomerization.

$$\text{Hexaene, } \% = k'_6 \cdot 3.722 - k'_5 \cdot 0.219$$
$$\text{Pentaene, } \% = k'_5 \cdot 1.437 - k'_6 \cdot 1.477$$
$$\text{Tetraene, } \% = k'_4 \cdot 1.65 - k'_5 \cdot 1.567 - k'_6 \cdot 0.282$$
$$\text{Triene, } \% = k'_3 \cdot 1.105 - k'_4 \cdot 0.879 + k'_5 \cdot 0.443 - k'_6 \cdot 1.534$$
$$\text{Diene, } \% = k'_2 \cdot 1.092 - k'_3 \cdot 0.573 - k'_4 \cdot 0.26 - k'_5 \cdot 0.026 - k'_6 \cdot 0.27$$

If the total fatty acids are determined by titration, the content of the different types of polyenoic fatty acids in equiv. per 100 equiv. total fatty acids is calculated by the following equations. These equations are based on the $E^{1\,\text{mequiv./l}}_{1\,cm}$-values in Table 1. k'_2, k'_3, k'_4, k'_5, and k'_6 are here given as $E^{1\,\text{mequiv./l}}_{1\,cm}$ of the sample instead of $E^{1\,g/l}_{1\,cm}$ as in the first set of equations.

$$\text{Hexaene, equiv./100 equiv.} = k'_6 \cdot 11.34 - k'_5 \cdot 0.669$$
$$\text{Petaene, equiv./100 equiv.} = k'_5 \cdot 4.75 - k'_6 \cdot 4.89$$
$$\text{Tetraene, equiv./100 equiv.} = k'_4 \cdot 5.42 - k'_5 \cdot 5.14 - k'_6 \cdot 0.921$$
$$\text{Triene, equiv./100 equiv.} = k'_3 \cdot 3.97 - k'_4 \cdot 3.16 + k'_5 \cdot 1.588 - k'_6 \cdot 5.52$$
$$\text{Diene, equiv./100 equiv.} = k'_2 \cdot 3.89 - k'_3 \cdot 2.04 - k'_4 \cdot 0.924 - k'_5 \cdot 0.961 - k'_6 \cdot 0.961$$

Table 3. Extraction of stearic acid from solutions with varying ethano concentration. 30 mg stearic acid.

Ethanol concentration % (v/v)	Number of determinations	ml pet.ether used for extraction	Recovery, %
25	6	20 + 10 + 10	90
40	2	20 + 10 + 10	94
50	5	20 + 10 + 10	98
50	12	10 + 10 + 10 + 10	99
62	3	20 + 10 + 10	97

RESULTS AND DISCUSSION

1. *Extraction.* Table 2 gives the results of experiments designed to show the reproducibility of the isolation of total fatty acids. It appears that the results are only slightly affected by variations in the sample size between 1 and 5 g. For a sample size of about 2 g the results can be reproduced within *ca.* ± 1 %. As the present experiments, besides extraction, include several other steps in the isolation of fatty acids, it can be concluded that the extraction method used is highly reproducible. It should be noted that ethanol:ether 3:1 in a volume 20—30 times that of the sample gives quantitative extraction of lipids [24]. In the experiments reported here the ratio between volume of ethanol:ether and sample varied from 12 to 60.

2. *Saponification and extraction of unsaponifiable and saponifiable fractions.* The experiments with stearic acid, the results of which are given in Table 3, indicate that the ethanol concentration of the saponification mixture should be about 50 % when the extraction of fatty acids is performed.

Other experiments showed that extraction of unsaponifiable matter before extraction of fatty acids results in a loss of about 0.5—2 % of stearic, oleic and linoleic acids, whereas linolenic acid is recovered 100 % in the fatty acid extract (*cf.* Table 4).

The results of recovery experiments, in which known amounts of fatty acids were saponified, extracted for unsaponifiable matter and extracted after acidification of the medium as described under methods, are given in Table 4. It appears that recovery is close to 100 % and that the methods used give highly reproducible results.

3. *The influence of saponification upon the determination of polyenoic fatty acids.* In order to determine the effect of saponification upon polyenoic fatty

Table 4. Recovery of fatty acids in acid extract after saponification and extraction of unsaponifiable fraction.

	Number of determinations	Recovery, %	SE	SD
Stearic acid	9	97.7	0.5	0.2
Oleic »	4	97.1	0.5	0.3
Linoleic »	2	98.1		
Linolenic »	6	100.5	1.0	0.4
Tristearin *	9	96.6	0.5	0.2
Olive oil *	5	98.0	0.7	0.3

* The recovery is calculated on a basis of a macrodetermination of the saponification number.

Table 5. The influence of saponification upon polyenoic fatty acids and soybean oil. All values are weight per cent of total fatty acids.

Fat	Saponification time, h.	% conj. diene before saponification	% conj. diene after saponification	% diene before saponification	% diene after saponification	% triene before saponification	% triene after saponification
Linoleic acid *	1	0.43	0.66	98.0	98.3	1.17	1.23
» »	1 ½	0.43	0.83	98.0	98.0	1.17	1.16
Linolenic acid **	1	1.29	2.86	−0.8	−1.7	99.6	98.2
» »	2	1.34	3.34	−0.8	−3.6	99.6	99.1
» »	2 ***	1.40	4.44	−0.8	−5.1	99.6	98.4
Soybean oil	1	0.27	0.60	51.9	52.2	7.73	7.78

* Hormel Foundation, containing only *cis*-isomers.
** Hormel Foundation, containing appreciable amounts of *trans*-isomers.
*** Allowed to stand hot for 1 ½ h after 2 h of saponification.

acids, different fats were saponified (see methods) for various length of time. The absorption spectra of non-isomerized as well as isomerized samples of the isolated fatty acids were determined and the composition was calculated. Table 5 gives the results of some experiments of this type with linoleic acid, linolenic acid and refined soybean oil. The content of conjugated constituents was calculated according to Brice and Swain [25] (see also Nørby [26]).

In another experiment (Table 6) six 2 g samples of calf liver homogenate (1:10 in water) were extracted, the extracts saponified for 1, 1 ½, or 2 h, and the proportion of conjugated and non-conjugated polyenoic fatty acids as well as total fatty acids determined as described. Besides the results given in Table 6 it was found that conjugated isomers with more than 3 double bonds were not present even after 2 h of saponification.

From Tables 5 and 6 it appears that small amounts of conjugated isomers are formed during saponification and that the degree of conjugation increases with increasing saponification time. The content of non-conjugated polyenoic fatty acids as determined by the isomerization method is, however, not signi-

Table 6. The influence of saponification time upon composition of fatty acids from calf liver.

Fatty acid *	Saponification time 1 hour		1 ½ hour		2 hours	
Conjugated dienoic	1.35	1.26	1.47	1.59	2.25	2.10
» trienoic	0.03	0.03	0.05	0.04	0.13	0.10
Dienoic	11.5	11.8	10.9	11.4	11.0	10.9
Trienoic	3.6	3.7	3.6	3.8	3.6	3.4
Tetraenoic	11.1	11.2	10.8	11.6	11.3	11.3
Pentaenoic	6.6	6.6	6.6	6.8	6.9	6.8
Hexaenoic	2.0	1.9	1.9	3.0	2.2	2.5
Total fatty acids **	8.78	8.72	8.65	8.51	8.28	8.45

* equiv./100 equiv. total fatty acids.
** mequiv./100 g liver (wet weight).

Table 7. Reproducibility of isomerization of pure acids and vegetable oils. Values for diene and triene are % of total fatty acids. Numbers in brackets are standard error of a single determination.

Sample	Sample weight * mg	No. det.	Diene %	Triene %
Linoleic acid	0.1—12	16	98.0 (1.2)	1.17 (0.07) **
Linolenic »	2.3— 3.5	9	—0.8 (0.4)	99.6 (0.6)
Soybean oil	1.7—40	19	51.8 (0.6)	7.68 (0.16)
Sunflower seed oil	3.3—17.8	5	59.1 (0.7)	0.106 (0.015) **

* The samples were weighed into small vials on a microbalance and isomerized. Samples weighing less than 0.5 mg were weighed as dilutions in lauryl alcohol.
** Determined according to Brice and Swain [25] and Nørby [26].

ficantly affected by saponification *. These observations agree well with that of Pikaar and Nijhof [12] who found that analysis of serum fatty acids after single and double saponification gave identical results.

From Fig. 2 in the paper of Pikaar and Nijhof [12] it appears that the absorbance at 233 mμ of serum fatty acids after saponification is about 15 % of that after isomerization. In a number of experiments on fatty acids from rat heart and rat plasma, which had a higher content of polyenoic fatty acids (see Table 8) than the fat analyzed by Pikaar and Nijhof [12], the author found the absorbance at 233 mμ after saponification to be 4—8 % of that after isomerization. The method of saponification and extraction of unsaponifiable and saponifiable fractions described here should probably be preferred to that of Pikaar and Nijhof [12] since it is more simple and produces less conjugation.

The results obtained in the experiments concerning the extraction and saponification step indicates that the methods used are well suited for the isolation of fatty acids from biological material prior to the determination of polyenoic fatty acids.

4. *Isomerization.* According to Herb and Riemenschneider [1] their microisomerization method requires only 1—10 mg of fat. In determinations on such a small amount of fat it is especially important that changes in the absorbance of the reagent itself are small and reproducible. In order to test the reproducibility of the blank (5.0 g reagent treated as described under methods), 8 series each consisting of 2 to 5 blanks were isomerized. After dilution to 25 ml with methanol the maximum difference in $E_{1\ cm}$ between the blanks in the same series was determined. The greatest difference between two blanks was 0.002, 0.003, 0.006, 0.016, and 0.035 at 375, 346, 315, 268, and 233 mμ. As extinction readings at 268 and 233 mμ usually are performed after dilution of the sample to 50 ml or more, the error introduced by variations in the reagent seems to be very small.

* The apparent decrease, with increasing saponification time, towards greater negative content of dienoic acid in the experiments with linolenic acid is an exception to this. If the specific extinction coefficient of the sample before saponification and before isomerization instead of that after saponification is used in the calculation of k_2', this decrease is not seen. This may indicate that the conjugated isomers formed by saponification of linolenic acid are unstable and during isomerization probably are converted to conjugated triene.

Table 8. Reproducibility of isomerization of fatty acids from egg yolk, rat plasma and rat heart. Numbers in brackets are standard error of a single determination.

Sample	Sample size	Number of duplicate det.	Content of polyenoic fatty acids * diene	triene	tetraene	pentaene	hexaene
			weight % of total fatty acids				
Egg yolk fatty acids	4—35 mg	10	10 (0.15)	0.8 (0.06)	2 (0.04)	0.4 (0.015)	1 (0.05)
			equiv./100 equiv. total fatty acids				
Rat plasma fatty acids	2—6 μequiv.	19	22 (0.55)	2 (0.27)	15 (0.42)	2 (0.21)	4 (0.28)
Rat heart fatty acids	*ca.* 3 μequiv.	21	20 (0.85)	0 (0.69)	16 (0.41)	2 (0.21)	11 (0.72)

* The composition of the fats analyzed is given in approximate values as a number of extracts from different yolks, plasmas and hearts were analyzed in duplicate.

The fatty acids were isolated and transferred to the isomerization tubes as described under methods p. 526.

The reproducibility of the isomerization method and the influence of sample size was tested in a number of experiments with pure fatty acids and vegetable oils (Table 7) and fatty acids from egg yolk, rat plasma and rat heart (Table 8). From the experiments with linoleic acid, vegetable oils (Table 7) and fatty

Table 9. Analysis of known mixtures of linoleic or linolenic acid and soybean oil by the method of Herb and Riemenschneider [1].

Expt. No. a)	Diene % calc.	found	Recovery, %	Triene % calc.	found b)	Recovery, %
1	88.5	88.1	99.5	2.59	2.55	98.5
2	85.6	85.9	100.4	2.98	2.87	96.3 c)
3	82.8	82.3	99.4	3.38	3.12	92.3 c)
4	82.4	82.2	99.8	3.44	3.40	98.8
5	77.1	76.0	98.6	4.14	4.17	100.7
6	76.8	76.3	99.3	4.21	3.96	94.1 c)
7	47.5	48.0	101.1	15.2	15.3	100.7
8	40.4	40.7	100.7	27.7	27.7	100.0
9	39.7	40.4	101.8	28.9	29.0	100.3
10	35.5	36.0	101.4	36.2	36.2	100.0
11	24.5	24.4	99.6	55.6	55.6	100.0
12	21.0	21.5	102.4	61.6	60.4	98.1
13	16.5	16.2	98.2	69.5	70.2	101.0
Average			100.2			99.8
SE			1.3			1.0
SD			0.4			0.3

a) 1—6: soybean oil + linoleic acid. 7—13: soybean oil + linolenic acid.

b) % triene in experiment 1—6 are calculated after correction for background absorption (Brice and Swain [25], Nørby [26]).

c) These samples, which were analyzed simultaneously, gave low triene values due to oxidation of the blank.

acids from egg yolk (Table 8) it is seen that the sample size may be varied over a wide range. Furthermore it appears from the results given in Table 8 that analysis of very small samples (*ca.* 3 μequiv. = *ca.* 1 mg) of complicated mixtures can be performed with considerable accuracy.

Results of experiments in which linoleic or linolenic acid were added to soybean oil and the mixtures analyzed are given in Table 9. Apart from experiments 2, 3, and 6, in which the blank was oxidized due to failure of the N_2-stream, the recovery was close to 100 %.

5. *Determination of total fatty acids.* The accuracy of the titration method described (p. 527) was determined by analysis of the results obtained in 19 duplicate titrations on 5 ml aliquots of fatty acid extracts from rat plasma and 21 duplicate titrations of fatty acids from rat heart. The standard error of a single titration was 1—3 %, the amount of fatty acid titrated being 2—6 μequiv.

It should be mentioned that titration of extracts from a saponification medium, to which pyruvic, lactic, β-hydroxybutyric, and citric acids had been added, gave values identical with the blank. This excludes these acids as sources of error in the determination of total fatty acids.

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