TABLE II

Properties of Buna-N Stock Plasticized with Some Diesteramide Plasticizers

Plasticizer N,N-bis(2-acetoxyethyl) amide of:	Tensile strength		Elongation		300% Modulus		Shore A hardness 10 sec	Weight loss	Brittle point	Volume change after 72 hr at 78F	Compatibility
	Unaged	Aged[a]	Unaged	Aged[a]	Unaged	Aged[a]					
	psi	*psi*	%	%	*psi*	*psi*		%	C	%	
Oleic acid (Ia)	2160	2010	790	740	760	1250	41	1.64	−40	28.5	I
Selectively hydrogenated cottonseed acids (VII)	2110	2030	810	540	690	1290	42	1.98	−44	27.5	C
Partially epoxidized cottonseed acids (XII)	2140	2200	900	640	550	1220	41	0.85	−40	30.1	C
Control (dibutylsebacate)	2170	2570	640	500	1080	1930	44	6.60	−50	20.0	C

[a] Aged for 48 hr in air oven at 212F.

the much lower volatility losses experienced with the diesteramides rather than to greater chemical stability. The diesteramides are not as effective in low-temperature performance as dibutyl sebacate nor are they as resistant to swelling in organic solvents, but they would be acceptable in applications where the ultimate performance in these two areas is not required.

ACKNOWLEDGMENTS

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Suitability of Lipid Extraction Procedures for Gas-Liquid Chromatography[1]

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Abstract

A comparison of two methods of extracting liver tissue lipids has been made using a limited amount of experimental material. The extracts prepared by a procedure using Bloor's reagent were more stable in storage, contained nearly 100% more total lipids and phospholipids, and produced more uniform and reproducible patterns on gas chromatographic analysis, than extracts of the same liver prepared by a diethyl ether Soxhlet extraction.

Introduction

THE HISTORY AND EVALUATION of extraction methods in lipid chemistry are thoroughly reviewed in the literature of the separate biological fields. Leach (1) summarized all of the studies on lipid content of microorganisms prior to 1906. Williams et al. (2) extracted lipids from microorganisms with 2 parts alcohol followed by 1 part ethyl ether in an extraction tower. An extensive review and comparison of lipid extraction techniques from microorganisms has recently been published by the U. S. Army Medical Research Laboratory, Fort Knox (3).

Bloor (4) thoroughly reviewed the extraction of lipids from animal tissues and proposed the use of ethanol-ether, 3:1 v/v, as an extraction reagent. Entenman (5) has more recently extensively reviewed the extraction of tissues.

The proximate analysis "ether extract" currently outlined in AOAC (6) originated at the Weende Experimental Station, Germany, in 1885 (7). In 1914 Walker and Bailey (8) introduced a simple, general extraction apparatus which has been the basic design for most of the micro-extraction equipment of the Soxhlet type currently used.

Where nutritional and biochemical studies are linked, it is the author's opinion that the Soxhlet procedure utilizing diethyl ether and a multisolvent extraction system should be compared not only with respect to total extractable lipids, but also with respect to the fatty acid patterns as determined by gas chromatography. This preliminary study undertakes to evaluate total lipid yield, alterations, and variabilities of two extraction procedures; viz., a procedure using a solvent system of 3:1 ethanol and ether (Method A), and a 4-hr Soxhlet extraction using diethyl ether (Method B).

Experimental Procedures

Eight male albino rats from our colony weighing 424–496 g, maintained on an adequate stock diet (9), were used as a source of liver in extraction procedure studies. Fresh liver weights ranged from 5.97–9.85 g. Following decapitation, the livers were removed, blotted, weighed, arbitrarily halved, and the halves

[1] Presented in part at the AOCS meeting in New Orleans, La., 1962.

TABLE I
Operating Conditions for Gas Liquid Chromatography

Sample	methyl esters of fatty acids
Carrier	argon, 20 psi. 107 ml/min
Cell voltage	600 v
Electrometer	gain 3
Flash heater	271C
Cell	260C
Column	180C
Packing	6 ft x ¼ in. I.D. Pyrex 12% ethylene glycol succinate polyester on 80/100 mesh Gas-Chrom P
Recorder	0–5 mv, 1 sec

weighed. Each half was then extracted by either a modified Bloor or a Soxhlet procedure.

Extraction Method A. The liver sample was ground in a macro-Virtis homogenizer for not more than 5 min in 95% ethanol. The homogenate was quantitatively transferred in ethanol to a 50 ml flask, brought to volume, and a 10-ml aliquot was transferred to a tared weighing container. The ethanol was evaporated at room temperature under a nitrogen stream. The dry matter sample was transferred to a vacuum oven and dried 4 hr at 60C. After drying, the samples were transferred to a desiccator, cooled, and weighed.

Another 10-ml aliquot of the homogenate was transferred to a 250 ml beaker; 3.3 ml of diethyl ether was added to give a 3:1 ethanol-ether mixture. Enough Bloor's reagent (3 parts 95% ethanol to 1 part diethyl ether) was added to give a ratio of 25 ml reagent per g of fresh liver. The mixture was brought to a slow rolling boil on a magnetic stirring hot plate. The extract was then decanted off through a filter paper into a 250 ml beaker and the process repeated twice more. The residue was rinsed with warm Bloor's reagent. The solvent was removed from the extract by placing the beaker over a steam bath. The last few ml of solvent were evaporated under a nitrogen stream. The residue was quantitatively transferred with petroleum ether into a 25-ml flask.

Extraction Method B. The liver sample was finely diced and the material quantitatively transferred in 95% ethanol to a beaker. Some extra ethanol was added to facilitate stirring. The ethanol was removed over a steam bath with final evaporation under nitrogen. The ethanol-treated liver was dried in a 60C vacuum oven 4 hr. Upon removal, the dried liver was pulverized and quantitatively transferred in diethyl ether to a thimble in a Bailey-Walker flask. The ether level was adjusted to ¼ in. below the siphon tip. The total volume of ether was 25–30 ml, depending upon siphon-tip length. Extraction was then made for 4 hr in Bailey-Walker apparatus. In unpublished data from this laboratory, Shue and Boehne in 1957 (10) found that 4-hr extractions of liver samples gave essentially the same gravimetric recovery values as extractions for longer periods. The ether was removed over a steam bath and the extract quantitatively transferred in petroleum ether to a 10 ml volumetric flask.

TABLE II
Total Lipid and Phospholipid Analysis of Liver Halves of the Same Origin (expressed as per cent of dry matter)

Rat no.	Initial analysis				41-day analysis	
	Method A		Method B		Method A	Method B
	Total lipid	Phospho-lipid	Total lipid	Phospho-lipid	Total lipid	Total lipid
1	23.2	11.5	10.5	5.5	22.5	10.1
2	20.4	10.7	11.3	8.0	20.6	10.0
3	24.6	11.1	10.8	4.4	.....	12.8
4	19.3	10.5	7.9	5.6	18.9	6.5
5	19.9	11.1	13.7	9.2	20.1	8.7
6	18.5	9.0	9.9	4.2	17.4	8.0
7	19.9	11.4	12.9	3.3	21.6	18.4
8	21.2	11.4	10.4	7.3	20.1	7.5
Average	20.9	10.8	10.9 [b]	5.9 [a]	20.2	10.3 [a]
Std. dev.	2.06	1.79	0.82	2.05 [a]	1.56	3.82 [a]

[a] Significant $P<0.05$
[b] Highly significant $P<0.01$

Preparation of Methyl Esters. Aliquots of the petroleum ether extracts resulting from both extraction procedures were interesterified by the method of Stoffel, et al. (11). The methyl esters of the fatty acids are taken up in petroleum ether in this procedure. The petroleum ether was removed under nitrogen and the esters taken up in *n*-hexane.

Chemical Analysis. Total lipids were determined on the petroleum ether extracts by the Bragdon procedure (12). Phospholipids were determined on the petroleum ether extracts, utilizing the molybdate-phosphorus color reaction for inorganic phosphorus (13).

Chromatographic Analysis. GLC analysis of the methyl ester preparations were made using a Model 10 Barber-Colman instrument with a 56 μc Radium-225 source detector. Operating conditions are shown in Table I.

Results and Discussion

Analysis for total lipids and phospholipids were

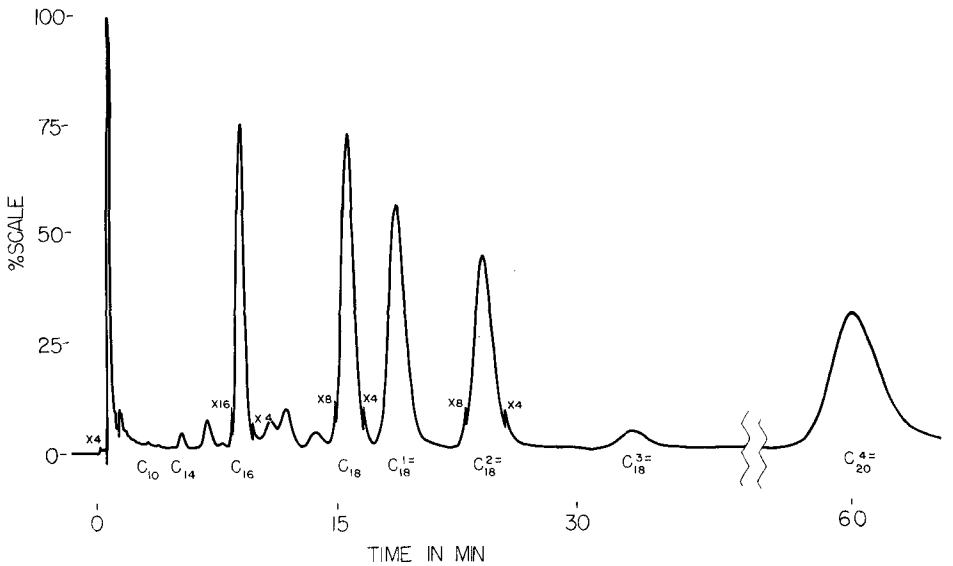

FIG. 1. Gas chromatographic analysis of the fatty acid methyl esters in Method A extract of rat liver No. 3. X indicates attenuation changes

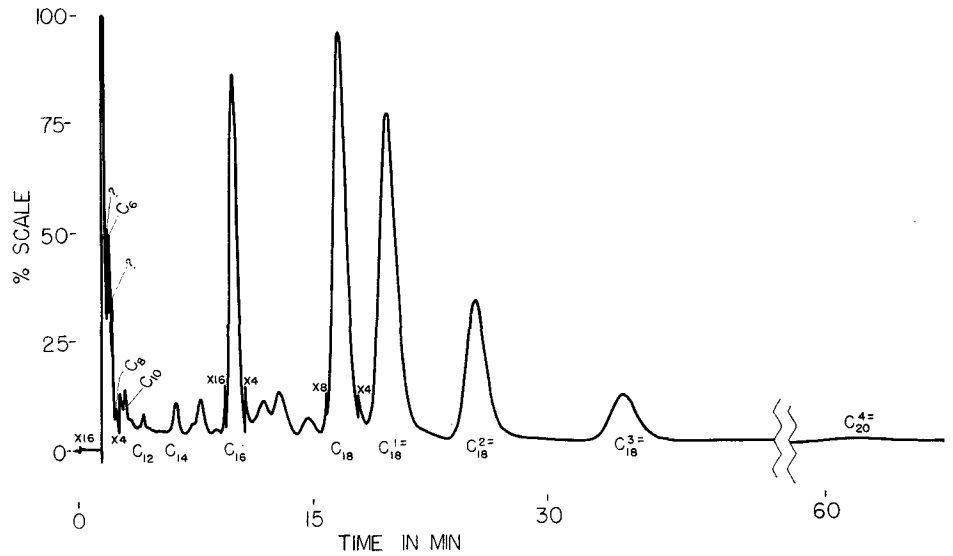

FIG. 2. Gas chromatographic analysis of the fatty acid methyl esters in Method B extract of rat liver No. 3. X indicates attenuation changes.

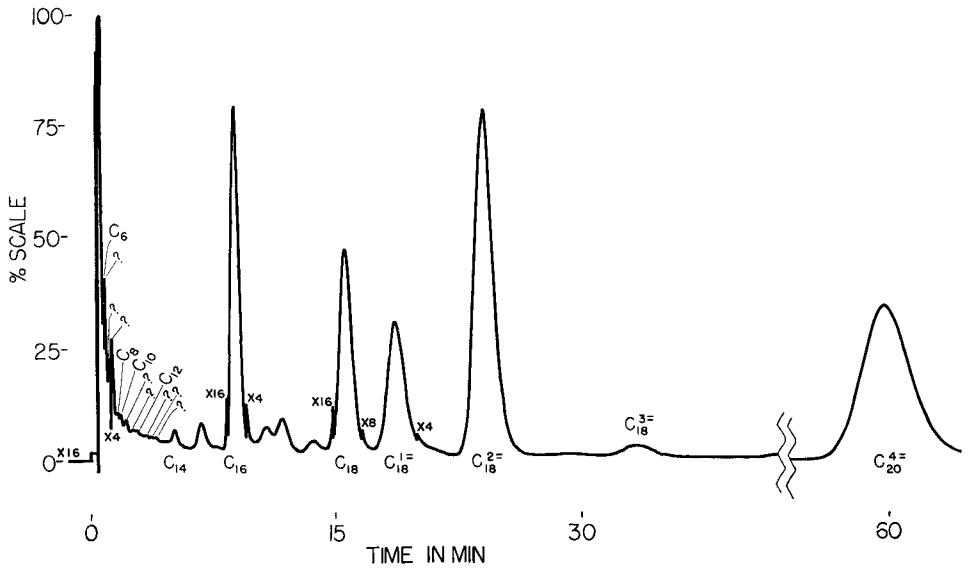

FIG. 3. Gas chromatographic analysis of the fatty acid methyl esters in Method B extract of rat liver No. 6. X indicates attenuation changes.

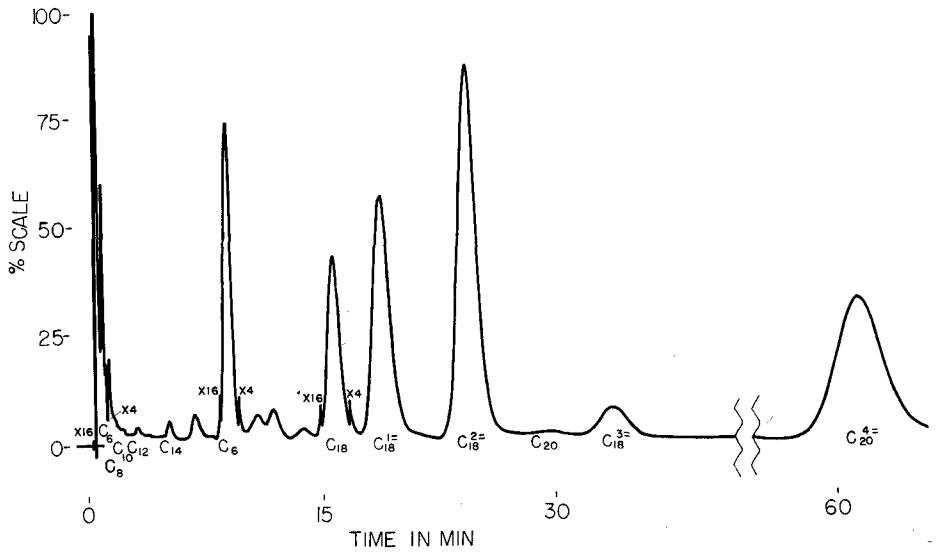

FIG. 4. Gas chromatographic analysis of the fatty acid methyl esters in Method A extract of rat liver No. 6. X indicates attenuation changes.

TABLE III

Gas Chromatographic Analysis—ester:stearate ratios in total liver lipids obtained by different extraction techniques

Rat no.	Method A						Method B					
	$C_{20}^{4=}$	$C_{18}^{3=}$	$C_{18}^{2=}$	$C_{18}^{1=}$	C_{16}	C_{14}	$C_{20}^{4=}$	$C_{18}^{3=}$	$C_{18}^{2=}$	$C_{18}^{1=}$	C_{16}	C_{14}
1	0.581	0.115	0.890	0.597	0.953	0.012	0.423	0.071	0.746	0.590	1.129	0.017
2	0.811	0.125	0.972	0.583	1.281	0.024	0.715	0.124	1.195	0.681	1.752	0.003
3	0.841	0.067	1.000	0.517	1.379	0.013	0.039	0.123	0.274	0.498	1.117	0.017
4	0.775	0.102	0.956	0.704	1.448	0.018	0.928	0.129	1.256	1.126	2.639	0.038
5	0.776	0.088	0.671	0.404	0.891	0.015	0.694	0.036	0.617	0.330	0.790	0.006
6	0.778	0.101	0.789	0.444	1.156	0.013	0.748	0.032	0.626	0.429	1.013	0.009
7	0.790	0.025	1.027	0.622	1.409	0.023	0.473	0.019	0.566	0.740	0.982	0.011
8	0.888	0.026	0.895	0.541	1.411	0.024	1.044	0.060	0.813	0.576	1.615	0.020
Max	0.888	0.125	1.027	0.704	1.448	0.024	1.044	0.129	1.256	1.126	2.639	0.038
Min	0.581	0.025	0.671	0.404	0.891	0.012	0.039	0.019	0.274	0.330	0.790	0.003

made within 48 hr following preparation of petroleum ether extracts. Forty-one days later a second total lipid analysis was made to determine the effect, if any, of storage at a temperature of 1C (see Table II).

In the initial analysis for per cent total lipids, the two methods exhibited the same relative amount of variability, but there was a highly significant difference in their average results. The average obtained by Method A was $20.9 \pm 2.06\%$, and by Method B, $10.9 \pm 0.82\%$. In the extraction of phospholipids, the two methods showed significantly different amounts of variability, and also significant differences in the averages. The average results by Method A and Method B were $10.8 \pm 1.79\%$ and $5.9 \pm 2.05\%$, respectively.

In the 41-day analysis, the two methods showed significantly different amounts of variability in the extraction of per cent total lipids. The averages were $20.2 \pm 1.56\%$ and $10.3 \pm 3.82\%$, respectively. The averages were significantly different. Method B extracts generally showed cloudiness, whereas Method A extracts did not. These latter data are of interest in light of the report of James in 1954 (14) that fatty acids can be stored for months in common solvents at +2C with no apparent decomposition. It appears from these data that Method B extracts had undergone some change during 41 days storage as indicated by the increase in variability exhibited, whereas Method A extracts remained relatively stable.

Representative chromatographs of the fatty acid methyl esters of the liver extracts are presented in Figures 1, 2, 3, and 4. Figures 1 and 4 are typical of those obtained from Method A extracts. Figures 1 and 2 are chromatograms of Method A and B extracts, respectively, of a single rat's liver. The outstanding feature of this pair of chromatographs is the virtual disappearance of the methyl arachidonate peak in Method B extract. Some extraneous materials were observed (question marks on Fig. 2) during the early minutes in the chromatograph of Method B extract.

Figures 3 and 4 represent the analysis of Method B and A extracts of rat liver. It is interesting that the methyl arachidonate peaks are prominent in both analyses, but an accumulation of extraneous material appeared (question marks on Fig. 3) in the low molecular weight peak region during the early minutes of the analysis of Method B extract. The proportion of methyl arachidonate was approximately the same in both extracts.

The appearance of these low molecular weight peaks, and the virtual disappearance of arachidonate, were not uniform throughout Method B extracts, nor did they necessarily occur together in the same chromatograph.

It was not possible to compare directly the data from the two extractions by calculating the relative areas (width at half-height × height = peak area) of all components because, when a large number of extraneous material peaks appear, the total area increases and the relative area of a given component is decreased; yet it may appear in the same molar concentration as in the comparable liver sample. To overcome this difficulty, stearate was selected as a reference point in each chromatograph, with the realization that its concentration may or may not be the same as in the counterpart extraction. A ratio of the areas of a number of fatty acid ester peaks to the area of stearate was calculated for each chromatograph (Table III).

Upon inspection of the range of observations, as indicated by the maximum and minimum values, Method B extracts exhibited a greater variation than Method A extracts of the same liver source. Statistical evaluation of these data is shown in Table IV.

There was no significant difference at the 5% level ($P<0.05$) between the ratio averages obtained from either extract. However, when considering the variability of the data which contributed to the ratio averages, the situation is quite different. In every case except two (linolenate:stearate, and myristate:stearate), the variability of contributing data was significantly greater for Method B. The myristate:stearate ratio standard deviation lacked one degree of freedom of being significantly greater for Method B.

TABLE IV

Statistical Evaluation of Ratios of Component Peaks to Stearate Peak Area

Fatty acid ester components	Ester: stearate area ratios		P<.05
	Method A	Method B	
$C_{20}^{4=}$	$\bar{x} = 0.780$ $\sigma = \pm 0.0895$	$\bar{x} = 0.633$ $\sigma = \pm 0.3168$	*
$C_{18}^{1=}$	$\bar{x} = 0.081$ $\sigma = \pm 0.0384$	$\bar{x} = 0.074$ $\sigma = \pm 0.0453$	
$C_{18}^{2=}$	$\bar{x} = 0.900$ $\sigma = \pm 0.1192$	$\bar{x} = 0.762$ $\sigma = \pm 0.3274$	*
$C_{18}^{3=}$	$\bar{x} = 0.552$ $\sigma = \pm 0.0969$	$\bar{x} = 0.621$ $\sigma = \pm 0.2427$	*
C_{16}	$\bar{x} = 1.241$ $\sigma = \pm 0.2182$	$\bar{x} = 1.380$ $\sigma = \pm 0.6038$	*
C_{14}	$\bar{x} = 0.018$ $\sigma = \pm 0.0052$	$\bar{x} = 0.015$ $\sigma = \pm 0.0109$	approaching

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