ABSTRACT

The first part of this thesis is concerned with a re-examination of the isolation of arachidonic acid/ester (20:4 5c 8c 11c 14c) from natural sources. Methyl arachidonate of 84% purity was obtained from pig liver by urea crystallisation and argentation chromatography. This ester was examined spectroscopically and in respect of its behaviour in heating. Pig liver also furnished 98% pure methyl arachidonate by fractional distillation followed by separating procedures previously used. An attempt was made to compare the efficiency of the separating procedures taking account of their cost. Finally, a procedure was recommended for the isolation of methyl arachidonate from pig liver.

The second part of the thesis deals with an examination of rapeseed oil for the presence of oxygenated fatty acids. Examination for the presence of furanoid acids involves the concentration of acids of this type as a result of urea crystallisation and argentation chromatography. Examination for the presence of epoxy and hydroxy acids in rapeseed oil methyl esters was based on chromatography. No oxygenated fatty acids detected in rapeseed oil in either of these studies.

THE ISOLATION OF PURE ARACHIDONIC ACID FROM NATURAL SOURCES AND EXAMINATION OF RAPESEED OIL FOR THE PRESENCE OF OXYGENATED FATTY ACIDS

being a thesis

presented by

Eltigani Sidahmed Mursi

to the

University of St. Andrews

in application for

The Degree of Master of Science

September 1980

To

my parents

and

my wife

To

my parents

and

my wife

DECLARATION

I hereby declare that this thesis is a record of the results of my own experiments, that it is my own composition, and that it has not previously been presented in application for a higher degree.

The research was carried out in the Department of Chemistry of the University of St. Andrews, under the supervision of Professor F. D. Gunstone, D. Sc., F. R. I. C.

CERTIFICATE

I hereby certify that Eltigani Sidahmed Mursi has completed eight terms of research work under my supervision, has fulfilled the conditions of Resolution of the University Court 1974 No. 2 (St. Andrews) and that he is qualified to submit the accompanying thesis in application for the degree of Master of Science.

Research Supervisor

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Professor F. D. Gunstone not only for the invaluable guidance and help throughout the period of my present studies, but also for his kind understanding and advice in private matters.

I am also deeply indebted to Dr. Charlie Scrimgeour for his helpful discussion at the bench.

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I appreciate the expert typing of the script by Miss Alison Thom.

Finally, I am indebted to Sudan Government for the grant and financial support.

CONTENTS

Part Two

ABBREVIATIONS

Ag^+ T. L. C.	Argentation thin layer chromatography
Ag^+ 1, 2, etc.	Argentation T. L. C. band one, two, etc.
b. p.	Boiling point
^{13}C n. m. r.	Carbon^{13} nuclear magnetic resonance
E. F. A.	Essential fatty acids
G. L. C.	Gas liquid chromatography
^{1}H n. m. r.	Hydrogen (proton) nuclear magnetic resonance
IR	Infra-red
M. L.	Mother liquor of urea crystallisation
PE	Petroleum ether and diethyl ether
SP 2340	Silicone polymer stationary phase (75% cyanopropyl)
Temp.	Temperature
T. L. C.	Thin layer chromatography
TMS	Trimethylsilyl
UV	Ultra-violet

ABSTRACT

The first part of this thesis is concerned with a re-examination of the isolation of arachidonic acid/ester (20:4 5c 8c 11c 14c) from natural sources. Methyl arachidonate of 84% purity was obtained from pig liver by urea crystallisation and argentation chromatography. This ester was examined spectroscopically and in respect of its behaviour in heating. Pig liver also furnished 98% pure methyl arachidonate by fractional distillation followed by separating procedures previously used. An attempt was made to compare the efficiency of the separating procedures taking account of their cost. Finally, a procedure was recommended for the isolation of methyl arachidonate from pig liver.

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PART ONE

THE ISOLATION OF PURE ARACHIDONIC ACID/ESTER FROM NATURAL SOURCES

1. Introduction

1.1 Importance of Arachidonic Acid

In 1918 Aron [1] concluded that fats have a specific nutritional value which cannot be provided by other dietary compounds. Burr and Burr [2] later showed that linoleic acid is essential to maintain the normal growth of rats and reported the effect of fat deficiency upon reproduction. They coined the new expression essential fatty acids (E. F. A.), which was historically applied to linoleic acid 18:2 (9c, 12c) and α-linolenic acid 18:3 (9c, 12c, 15c). Essential fatty acids are those preventing or relieving symptoms of fat-deficiency in animals.

Arachidonic acid (I, Scheme 1.1) belongs to the same family of polyene acids as linoleic acid (II, Scheme 1.1)

I $CH_3(CH_2)_4(CH{=}CH.\ CH_2)_4(CH_2)_2COOH$

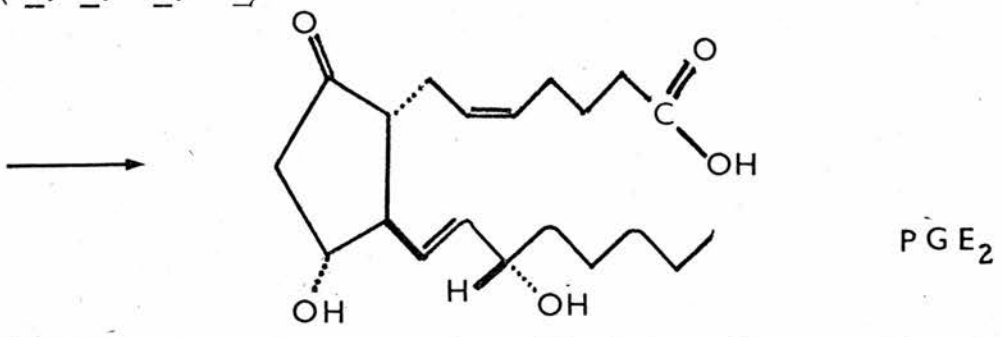
II $CH_3(CH_2)_4(CH{=}CH.\ CH_2)_2(CH_2)_6COOH$

SCHEME 1.1: The structures of 20:4 (5c, 8c, 11c, 14c) and 18:2 (9c, 12c) fatty acids.

Linoleic acid (II, Scheme 1.1) is converted by desaturation and elongation processes to arachidonic acid (I, Scheme 1.1), which is itself the precursor of several physiologically important compounds including the prostaglandins (Scheme 1.2) and thromboxanes.

18:2 (9c, 12c) ——> 18:3 (6c, 9c, 12c) ——> 20:3 (8c, 11c, 14c) ——>

20:4 (5c, 8c, 11c, 14c) ——>



PGE_2

SCHEME 1.2: Enzymic conversion of linoleic acid to arachidonic acid and a typical prostaglandin (PGE_2).

The ratio of the 20:3 (n-9) triene acid (derived from oleic acid by similar desaturation and elongation precesses) to arachidonic acid 20:4 (n-6) is now used as the simplest index of EFA deficiency in human [3].

Growing interest in the chemistry and biochemistry of arachidonic acid warrants a re-examination of the methods of obtaining this compound by isolation from natural sources or by chemical synthesis.

1.2 Isolation from Natural Sources

In 1902 Hartley [4] reported the presence of arachidonic acid in liver lipids. It is now perhaps the best known higher polyunsaturated fatty acid, because of its relatively widespread occurrence in animals. It occurs as a trace component in some glycerides and as a minor or major component in many phosphoglycerides.

Although arachidonic acid occurs so widely its isolation is comparatively difficult for two main reasons : (a) It is usually present in natural lipids as a minor component (up to 10%) and co-occurs with other polyene acids with similar physical properties and (b) it is

very susceptible to autoxidation.

The following materials have been used or may be considered as a starting point for the isolation of arachidonic acid :

(a) Beef adrenals, the lipids of which contain about 9% of arachidonic acid [4].

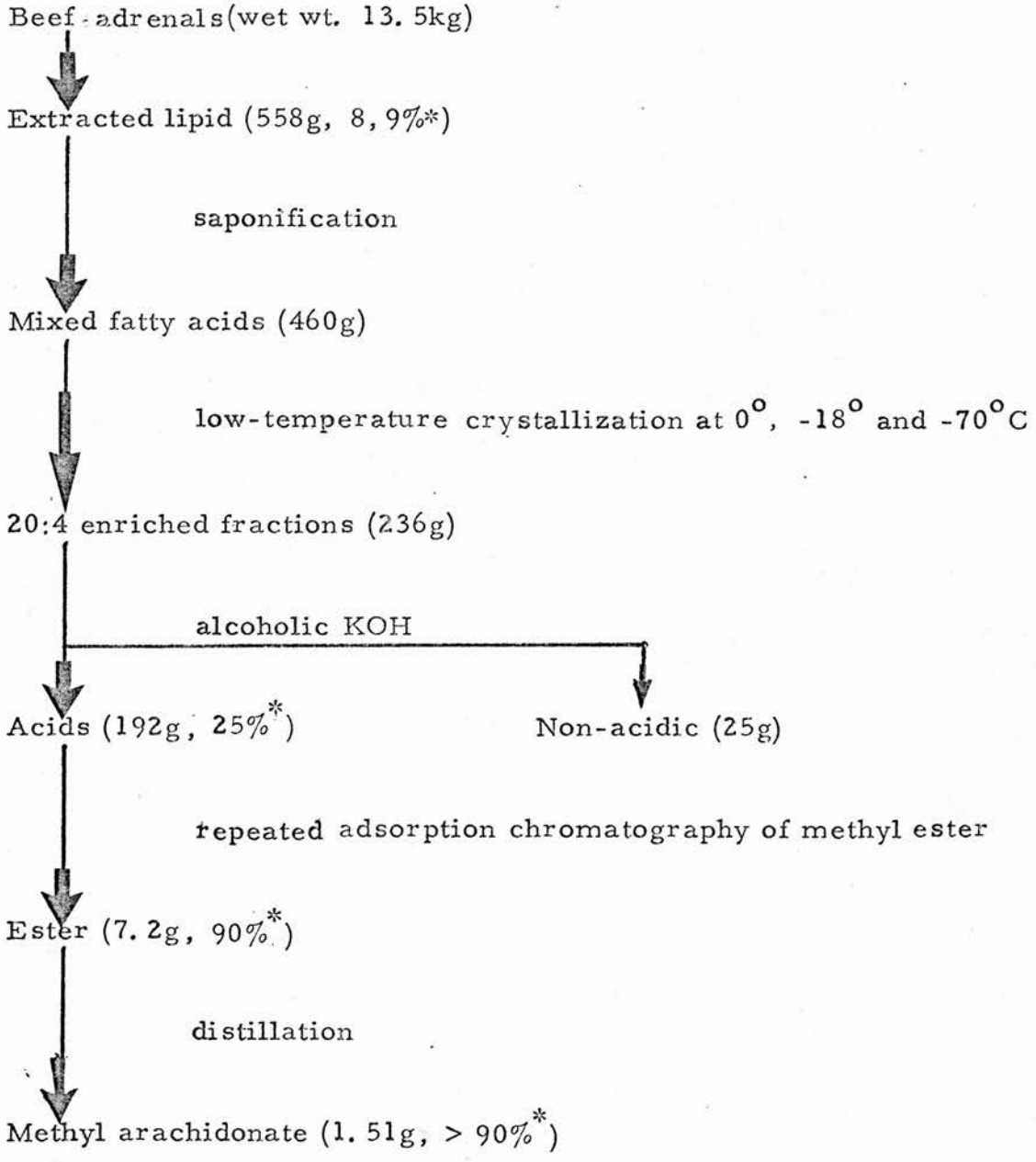
(b) Liver phospholipids with 4 to 5% of arachidonic acid [5].

(c) Other minor sources of arachidonic acid include egg yolk, beef heart, bovine testes and some fish liver oils.

(d) Moss lipids contain up to 34% of this acid. Its presence in fern fronds has been reported by Haigh who found 26% in lipids from harts tongue. Nevertheless, this is unlikely to be a useful source of arachidonic acid [6].

The isolation of arachidonic acid from animal tissues involves the working up of large amounts of materials and is effected by a combination of separation techniques.

By the procedure outlined in (Scheme 1. 3) Herb, Riemenschneider and Donaldson [4] isolated arachidonic acid of high purity (1. 5g > 90% pure) from beef adrenals (13. 5 kg wet weight). They employed low-temperature crystallisation, adsorption chromatography and distillation. The purity of the product was determined by iodine value, spectrophotometric examination after alkali isomerization, saponification value, mean molecular weight, x-ray diffraction pattern, and melting point of a completely hydrogenated portion.

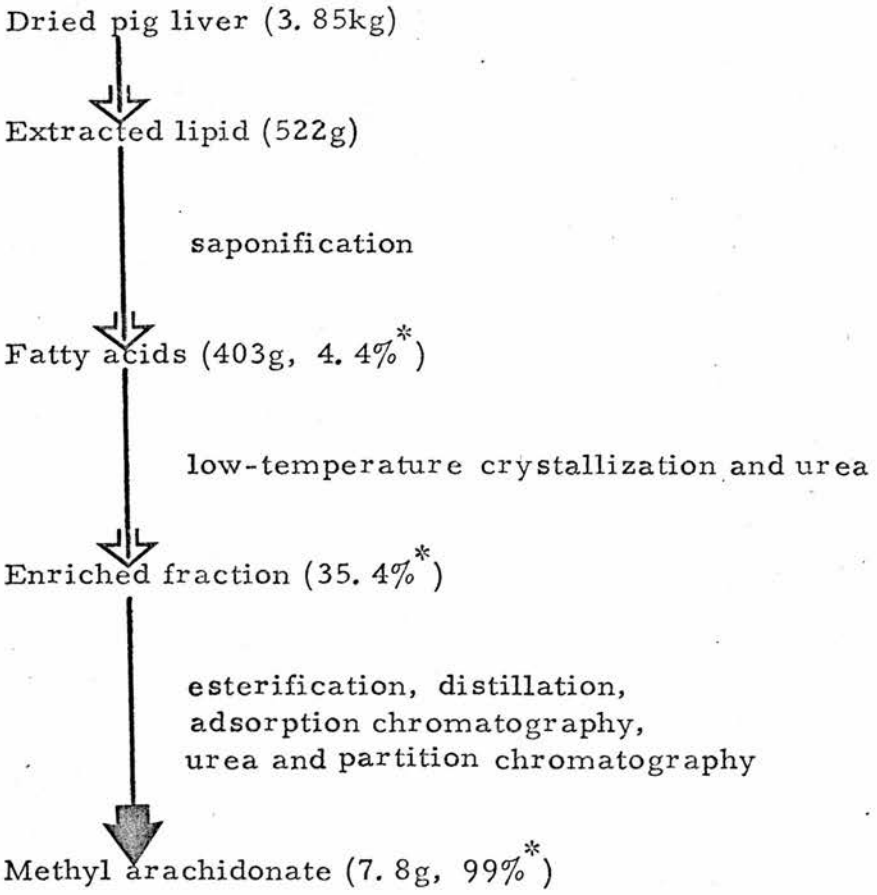


SCHEME 1.3 : Isolation of arachidonic acid/ester from beef adrenals by Herb, Riemenschneider and Donaldson.

* indicates concentration of arachidonic acid/ester.

Privett, Weber and Nickell[5] later described a procedure for isolation of methyl arachidonate (~99%) from dried pig liver (Scheme 1.4). The fatty acids obtained from the extracted liver lipid (20:4 ~ 4.4%) were first enriched in arachidonic acid by low-temperature crystallization followed by urea fractionation (20:4 ~ 34.4%).

The acids were then esterfied and distilled under reduced pressure. The C_{20} distillate, further purified by adsorption chromatography, urea fractionation and partition chromatography, furnished methyl arachidonate of 99% purity as determined by gas-liquid chromatography, paper chromatography, alkali isomerization and iodine value. Many of these separation procedures were repeated several times to give the necessary purification so that the whole sequence is quite long. The authors claim to recover 44% of the arachidonic acid/ester at this high level of purity.



SCHEME 1. 4: Isolation of arachidonic acid/ester from pig liver by Privett, Weber, and Nickell

* indicates concentration of arachidonic acid/ester.

1.3 Synthesis of Arachidonic Acid

Polyenoic acids can be synthesised by a number of procedures including the use of acetylenic intermediates, Wittig reaction, and some other less common routes. Arachidonic acid is most commonly obtained by the first of these procedures and its synthesis via icosa-tetra-ynoic acid is outlined in (Scheme 1.5).

$$CH_3(CH_2)_4C\equiv CCH_2Br \xrightarrow{i,\,ii} CH_3(CH_2)_4(C\equiv CCH_2)_2Br \xrightarrow{i,\,ii}$$
$$\longrightarrow CH_3(CH_2)_4(C\equiv CCH_2)_3Br \xrightarrow{iii} CH_3(CH_2)_4(C\equiv CCH_2)_4(CH_2)_2COOH \xrightarrow{iv} CH_3(CH_2)_4(CH{=}CHCH_2)_4(CH_2)_2COOH$$

Reagents : i, $BrMg\,C\equiv CCH_2OMg\,Br$, CuCl; ii, PBr_3;

iii $BrMgC\equiv C(CH_2)_2COO\,MgBr$, CuCN; iv, H_2, Lindlar's catalyst

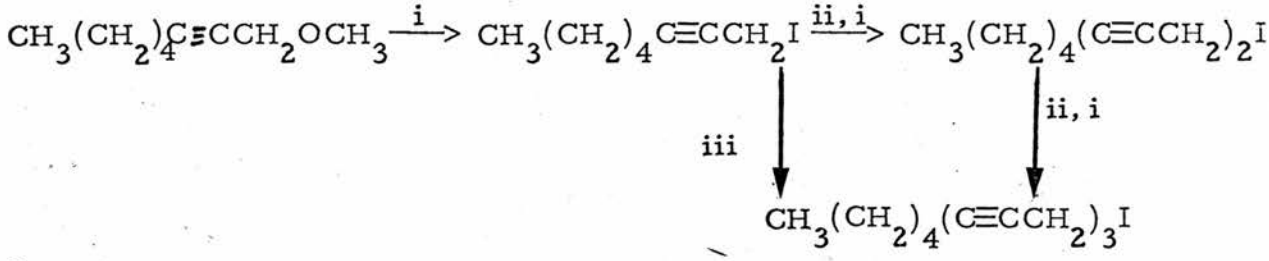
SCHEME 1.5 : Synthesis of arachidonic acid (Osbond, 7).

An advantage of this acetylenic route is that the poly-ynoic acid intermediate is a crystalline solid which can be purified by crystal-isation before being submitted to partial reduction using Lindlar's catalyst which is both stereospecific (giving only the cis alkene) and selective (reducing triple bonds but not double bonds).

In Osbond's synthesis (Scheme 1.5) propargyl alcohol ($HC\equiv CCH_2OH$) serves as a useful source of two of the unsaturated centres, and of the methylene group separating successive units of unsaturation. The intermediate alcohols have to be converted to bromides before reacting with further propargyl alcohol.

Wallat and Kunau [8] (Scheme 1.6) improved the synthesis by employing iodides (made from the alcohols via their methanesulphonyl

esters) which can then be converted to poly-ynoic acids by Osbond's procedure.



Reagents: i, CH_3COI; ii, $BrMgC{\equiv}CCH_2OCH_3$; iii, $BrMgC{\equiv}CCH_2C{\equiv}CCH_2OCH_3$

SCHEME 1.6 : Synthesis of substituted propargyl iodides (Kunau, 8).

The synthetic procedures are lengthy and do not easily lend themselves to large scale production. A considerable amount of development would have to be undertaken before synthesis of arachidonic acid would compete commercially with the alternative approach of isolation from natural sources, and the present study has been confined to an examination of isolation procedures.

2. RESULTS AND DISCUSSION

Two procedures are now described for the isolation of arachidonic acid/ester from pig liver. Both involve urea crystallisation and argentation chromatography. They differ, however, in that one procedure does not include distillation and the other does.

2.1 Isolation of arachidonic acid/ester from pig liver by urea crystallisation and argentation chromatography (excluding distillation):

The isolation procedure is summarised in Scheme 2.1 which also shows the weight of each significant fraction and the content of methyl arachidonate. By this sequence of separations methyl arachidonate of 84% purity was obtained.

Fresh pig liver (995g wet weight) was extracted with chloroform-methanol to give a lipid-enriched extract (65.8g). Treated with methanolic sulphuric acid, this furnished crude methyl ester (48.5g) which was freed of cholesterol by chromatography on silica. The recovered esters (37.5g) were analysed by gas liquid chromatography and shown to contain 9.6% of methyl arachidonate. The total amount of this ester present at this stage is therefore 3.6g. The composition of the mixed liver ester is given in Table 2.1. Structural assignments are based on gas liquid chromatographic retention behaviour (expressed as equivalent chain length - ECL), using the data reported by Jamieson [9]. The liver esters are rich in saturates (40%) and in monoene and diene esters (30%). The desired tetraene (9.6%) is accompanied by significant amounts of penta- and hexa-ene esters (16%).

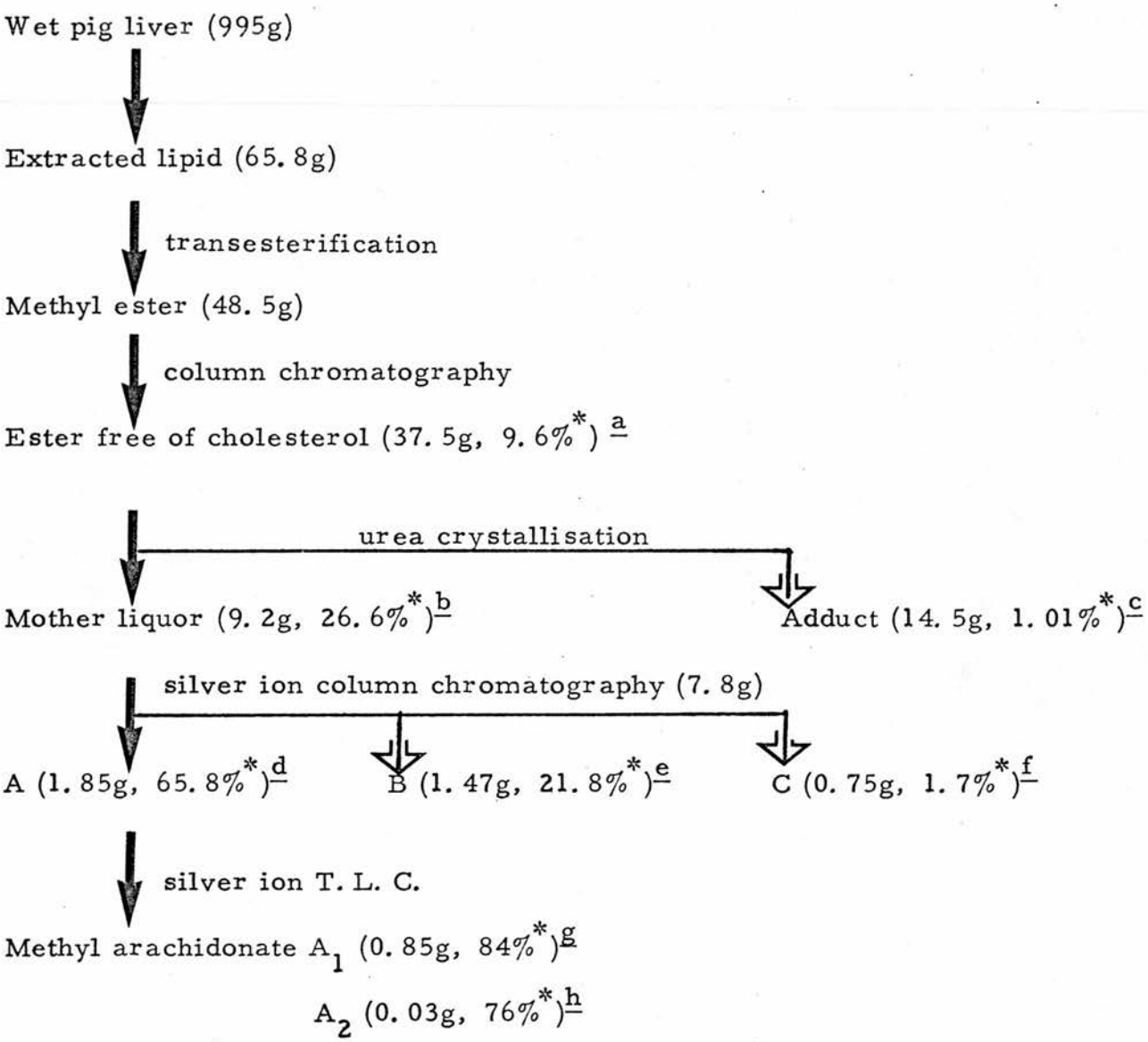
In an attempt to concentrate the polyene esters, the mixed esters were first crystallised from methanol in the presence of urea. The adduct con-

tained most of the saturated, monoene, and diene esters. The mother liquor (9. 2g) was enriched in polyene esters with arachidonate at the 26. 6% level (Table 2. 1).

A small sample (100mg) of this material, separated by silver ion thin layer chromatography, furnished a band containing methyl arachidonate (0. 02g, 86. 9%) with 18:3 n-6 (3. 6%), 18:3 n-3 (4. 7%), 20:3 n-6 (2. 5%) and 22:4 n-6 (0. 8%) as the major impurities.

The bulk of the concentrate (7. 8g) was purified in two batches on silver ion-silica columns. The esters were eluted with PE20 containing increasing proportions of methanol, and the separation was monitored by Ag^+ T. L. C. Three combined fractions were obtained: A (1. 85g, 65. 8%), B (1. 47g, 21. 8%) and C (0. 75g, 1. 7%). Full analytical details are listed in Table 2. 1. Fraction A, further purified by Ag^+ T. L. C. (10 plates), gave fractions A_1 (0. 85g, 84. 2%) and A_2 (0. 03g, 76. 1%) (Table 2. 1).

This series of separations would have given about 1. 0g of 84% methyl arachidonate if all the material (9. 2g) had been handled. This corresponds to 27. 8% recovery of the arachidonate originally present. The major impurities in the concentrate are C_{18} trienes (6. 4%), C_{20} trienes and pentaenes (4. 1%), and C_{22} esters with four, five, and six double bonds (3. 1%).



Scheme 2. 1 : Isolation of methyl arachidonate of 84% purity by urea crystallisation and argentation chromatography. (Full details of fractions a to h are given in table 2. 1.)

* Indicates concentration of arachidonic acid/ester.

TABLE 2.1

Component esters (% wt) of lipids obtained from pig liver and several fractions obtained therefrom :-

Esters	a	b	c	d	e	f	g	h
16:0	15.7		21.3	0.3	0.7			
16:1	1.2	0.5		0.2	1.2			0.1
18:0	23.1	0.8	33.2	0.2	1.7			0.1
18:1	16.9		30.2	0.6	2.5			0.1
18:2 (n-6)	11.7	13.0	10.9	7.9	37.2	0.8	0.8	1.8
18:3 (n-6)		1.4		1.8	1.7		2.9	0.6
18:3 (n-3)	1.1	1.6	0.8	2.3	2.0		3.5	0.9
20:3 (n-6)	0.8	2.4	0.4	2.7	3.5		3.9	0.9
20:4 (n-6)	9.6	26.6	1.0	65.8	21.8	1.7	84.2	76.1
20:5 (n-3)	5.8	17.8	0.2	6.6	12.2	34.9	0.2	2.5
22:4 (n-6)	0.3	1.2		1.1	0.5		1.6	0.8
22:5 (n-3)	3.2	8.1	1.1	4.5	5.6	22.1	0.4	2.0
22:6 (n-3)	6.9	23.0	0.1	2.0	2.3	37.6	1.1	8.7
Others	3.7	3.6	0.8	4.0	7.1	2.9	1.4	5.4

a: total pig liver ester

b: mother liquor from urea crystallisation

c: adduct from urea crystallisation

d, e and f: fractions A, B and C obtained by silver ion column chromatography

g and h: fractions A_1 and A_2 obtained by silver ion T. L. C.

2. 2 Isolation of arachidonic acid/ester from pig liver by fractional distillation, urea crystallization, and argentation chromatography :-

The general procedure of this method is outlined in Scheme 2. 2 which gives the weight and methyl arachidonate concentration in the main fractions.

Fresh wet pig liver (1800g) was extracted to obtain lipid (86g) which was transesterified and analysed by gas liquid chromatography (Table 2. 2). The concentration of methyl arachidonate in the crude methyl ester (72g) is 11. 4%.

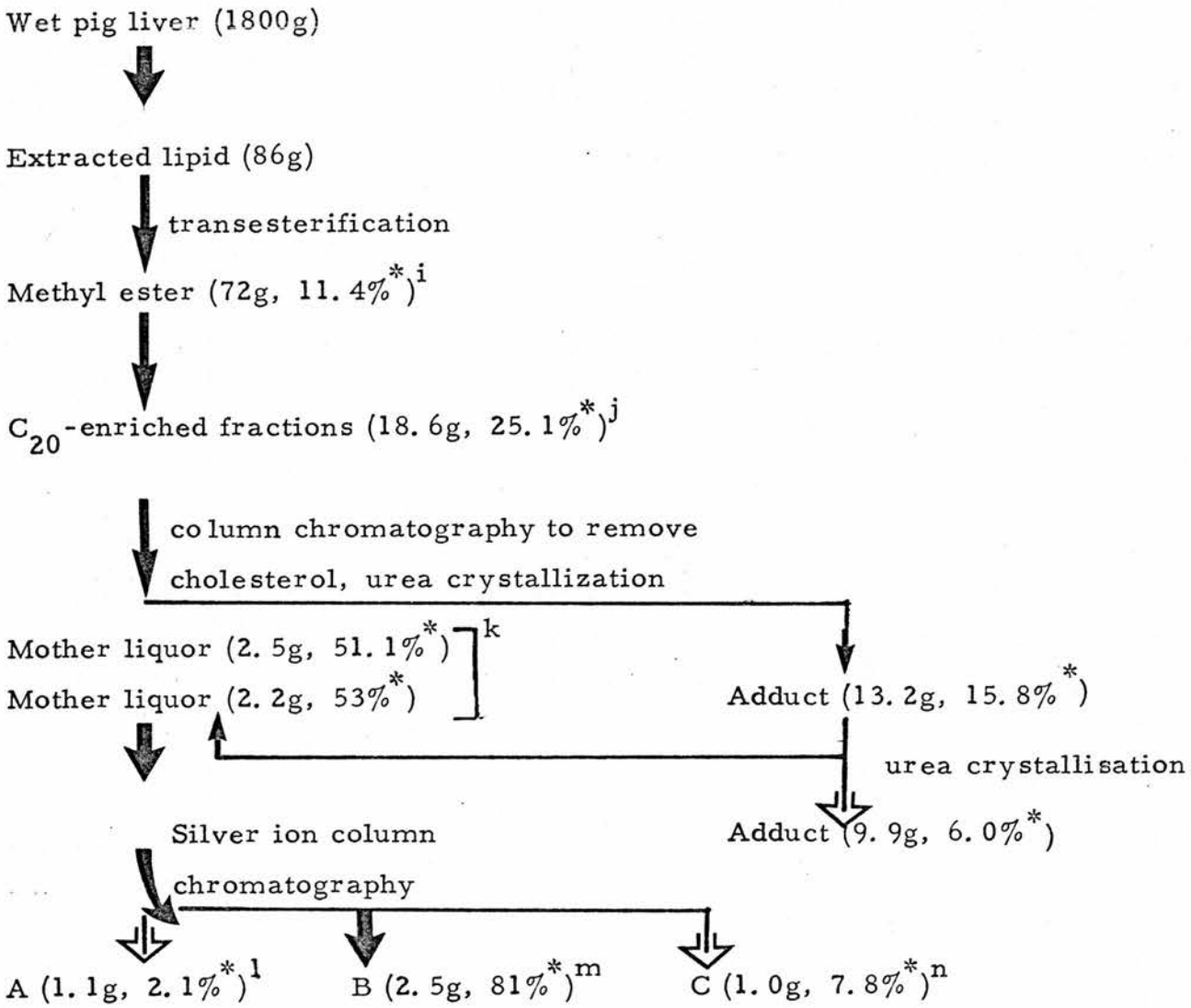
The crude ester was submitted to fractional distillation and two fractions rich in methyl arachidonate were combined to give a fraction (18. 6g) containing C_{18} (54. 6%), C_{20} (32. 3%) and C_{22} (10. 5%) esters with a concentration of methyl arachidonate of 25. 1%. The distilled esters were freed from cholesterol by column chromatography on silica and the recovered ester (17. 5g) was crystallized from methanol in the presence of urea. The mother liquor (2. 5g) contained 51% of methyl arachidonate. The adduct (13. 2g) still contained 15. 8% of this ester and was therefore again crystallized from urea to give more liquor (2. 2g) enriched in methyl arachidonate (53%). These two mother liquors were combined. In addition to the arachidonate (52%) unsaturated C_{18} esters (12%) other C_{20} polyenes (14%), and C_{22} polyenes (21%) were also present.

The bulk of this material (4. 7g) was run through a silver ion column to furnish three main fractions. The first one A (1. 1g) contained C_{18} esters (78%) and methyl arachidonate (2. 1% only). The main fraction B (2. 5g) containing methyl arachidonate (81%) was accompanied by C_{18} triene esters (5%).

C_{20} triene and pentaene esters (10%) and C_{22} polyene esters (4%). The next fraction C (1.0g) contained little arachidonate (8%) and was mainly 20:5 (33%), 22:5 (9%) and 22:6 (49%) esters. Because we did not determine the amount of ester excluding cholesterol it is not possible to calculate the yield of recovered methyl arachidonate, but we believe this to be about 25% as in the preceding and the following experiments.

Using a similar procedure yet another batch of pig liver (1890g) was transesterified and freed from cholesterol to obtain mixed methyl esters (77g, 9.6%). After distillation, urea crystallization and silver ion column chromatography two enriched fractions were recovered: 0.86g (96.5%) and 1.28g (87%). These together represent 1.94g of methyl arachidonate and a recovery of 26.3%.

An attempt was finally made to purify the combined samples of methyl arachidonate p (2.50g + 0.86g + 1.28g = 4.6g, 88%) by argentation column chromatography. Fractions r (0.12g, 25%), s (0.63g, 72%) and t (3.5g, 98%) were obtained (see Table 2.3 for full analysis). The 98% methyl arachidonate represents 3.43g which corresponds to about 25% of that originally available from the two liver samples.



Scheme 2.2 : Isolation of arachidonic acid/ester of 81% purity by fractional distillation , urea crystallisation and argentation chromatography.

* indicates concentration of arachidonic acid/ester.

The letters i to n refer to the analytical data in Table 2.2.

TABLE 2.2

Component esters (% wt) of lipids obtained from pig liver and several fractions obtained therefrom :-

Esters	i	j	k	l	m	n
16:0	15.7	0.2	-	-	-	-
16:1	1.2	0.2	0.1	0.5	-	-
18:0	23.1	22.6	-	5.1	-	-
18:1	17.0	16.0	1.2	15.2	-	-
18:2 (n-6)	16.1	13.8	9.4	57.8	0.2	-
18:3 (n-6)	-	0.7	0.7	2.4	1.4	-
18:3 (n-3)	1.4	1.5	1.1	-	2.1	0.1
20:3 (n-6)	0.7	1.9	2.0	2.4	3.4	0.1
20:4 (n-6)	11.4	25.1	51.8	2.1	81.1	7.8
20:5 (n-3)	2.2	5.3	11.6	1.5	6.6	33.0
22:4 (n-6)	0.2	0.6	0.5	1.5	1.0	0.3
22:5 (n-3)	2.5	3.3	4.3	3.8	3.4	9.1
22:6 (n-3)	6.3	6.6	16.6	3.4	-	48.8
Others	2.2	2.2	0.7	4.9	0.8	0.8

i : total pig liver crude ester.

j : C_{20}-enriched distilled fractions.

k : combined mother liquor from urea crystallization.

l, m and n : the main fractions obtained by silver ion column chromatography.

TABLE 2.3

The purification of combined samples of methyl arachidonate rich fractions by argentation column chromatography :-

Esters	p	r	s	t
18:2 (n-6)	0.1	1.6	0.1	-
18:3 (n-6)	1.8	17.9	6.6	0.2
18:3 (n-3)	2.5	22.6	10.7	0.3
20:3 (n-6)	1.8	25.8	4.9	0.2
20:4 (n-6)	87.7	24.8	72.4	98.1
20:5 (n-3)	2.6	4.0	0.2	0.3
22:4 (n-6)	1.0	2.2	3.6	0.7
22:5 (n-3)	1.7	-	0.4	0.2
22:6 (n-3)	0.2	-	-	-
Others	0.6	1.1	1.1	-

p : total combination of the enriched fractions of methyl arachidonate isolated from the two batches.

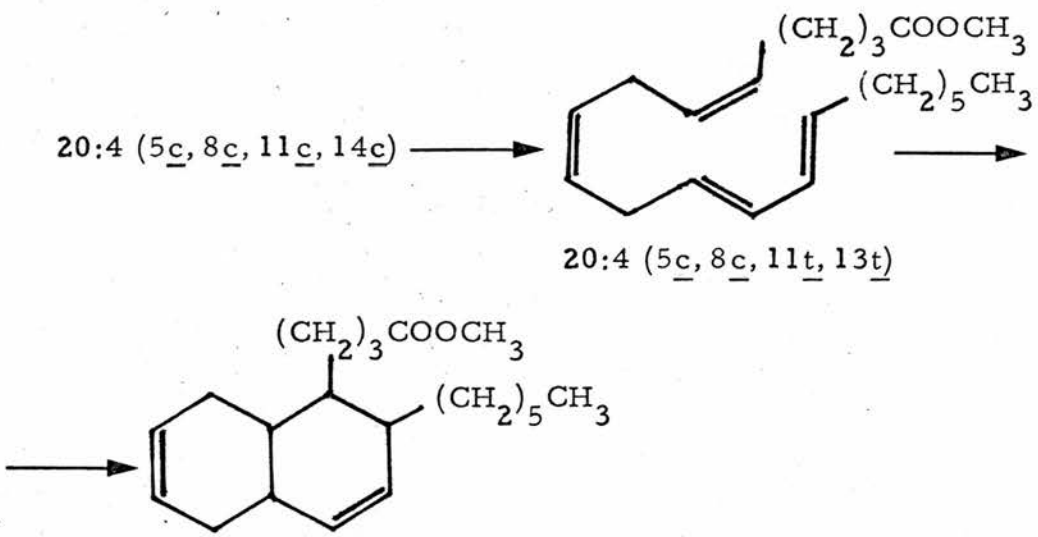
r, s and t : fractions obtained from silver ion column chromatography for combination (p).

2. 3 Some Properties of Methyl Arachidonate

A– Effect of heat :-

Methyl arachidonate purchased from suppliers of fatty acids is probably prepared from pig liver by isolation procedures which include distillation. This involves the risk of stereomutation, double bond migration and cyclisation though purification processes carried out after heating could possibly remove any artefacts.

Thermal treatment of polyene ester such as methyl arachidonate can produce cyclised compounds by an intramolecular Diels-Alder reaction following appropriate double bond migration and stereomutation (scheme 2. 3):



Scheme 2. 3 Possible cyclisation of arachidonic acid/ester.

To investigate this matter further we have carried out the following studies :

(a) Comparison of purchased methyl arachidonate (93%) with a sample (84%) isolated from pig liver without distillation as already described in Scheme 2. 1.

(b) Comparison of our 84% methyl arachidonate (Scheme 2. 1) before and after heating to 222°C for two hours in an atmosphere of nitrogen.

In an attempt to concentrate artefacts the prepared and the purchased methyl arachidonates were examined by urea fractionation and by silver ion thin layer chromatography.

By urea fractionation the prepared arachidonate (84%) was divided into an adduct containing 84% of 20:4 and a mother liquor with 80% of this ester. The mother liquor components gave expected ECL in G. L. C analysis (Table 2. 4) and there was no strong evidence of an unusual component which might have been cyclised ester concentrate in the mother liquor. This was equally true of purchased ester of slightly higher purity (Table 2. 5).

Silver ion chromatography might be expected to concentrate compounds with trans unsaturation or cyclic esters with less unsaturation than the arachidonate in fractions other than that rich in the all-cis 20:4 ester.

The prepared arachidonate was separated into three fractions containing 60%, 78% and 93% of methyl arachidonate, most of the other components in each fractions had ECL associated with impurities expected from the original arachidonate (84%). In addition, the first fraction in particular was very small. The purchased arachidonate (93%) also gave three fractions with 22%, 93% and 99% of arachidonate. The very small first fraction and the second fraction contained a component of ECL 21. 41 (19% in fraction one) and 21. 52 (3% in fraction two) which corresponds with the 20:3 (n-9) ester though this had not been observed in the original material. The infra red spectrum of the first fraction (ECL 21. 41)

showed no unexpected absorption and there was no evidence of trans unsaturation.

A sample of the isolated arachidonate (84% was heated in a nitrogen atmosphere at 222°C for two hours. The product was examined by urea fractionation and by silver ion chromatography (Table 2. 6). The liquor contained only 60% of arachidonate along with other esters having 4-6 double bonds (27%) and other minor components. Silver ion chromatography furnished two bands with 53% and 90% of arachidonate. When hydrogenated the first band gave only n-saturated acids. There was no evidence that heating had changed the arachidonate to any large extent.

TABLE 2.4

Methyl arachidonate isolated without heating

Esters	Sample	Urea adduct	Mother liquor	Ag^+ 1	Ag^+ 2	Ag^+ 3
16:0				2.4		
16:1				0.7		
18:0				0.6		
18:1	0.1	0.1	0.8	19.4	0.1	
18:2 (n-6)	0.8	1.0	0.3	0.7	2.0	
18:3 (n-6)	2.9	2.9	4.8		2.4	0.9
18:3 (n-3)	3.5	4.2	1.3	8.3	6.0	2.3
20:3 (n-6)	3.9	4.4	2.2		8.3	0.7
20:4 (n-6)	84.2	84.0	80.1	59.5	78.0	93.2
20:5 (n-3)	0.2	1.9	1.3			0.8
22:4 (n-6)	1.6	0.2	0.4		1.1	1.2
22:5 (n-3)	0.4	0.4	0.5			
22:6 (n-3)	1.1	0.1	7.4			
Others	1.3	0.8	0.9	8.4 (a)	2.4 (b)	0.9

(a) ECL 22.84 (3.3%) at 24.51 (4.8%) (ECL of methyl arachidonate 22.76).

(b) ECL 21.36 (1.7%) (ECL of arachidonate 22.29).

TABLE 2.5

Purchased methyl arachidonate

Esters	Sample	Urea adduct	Mother liquor	Ag^+ 1	Ag^+ 2	Ag^+ 3
16:0				0.4		
16:1				0.4		
18:0						
18:1	0.1	0.1	0.2	2.7	0.1	
18:2 (n-6)	4.6	5.3	2.8	54.5	3.9	
18:3 (n-3)	0.1		0.2		0.1	
20:3 (n-9)				19.6	2.8	0.2
20:4 (n-6)	93.3	92.4	94.6	22.3	92.9	98.8
20:5 (n-3)	0.4	0.1	0.3		0.1	0.4
Others	(a) 1.5	(b) 2.1	(c) 1.9	0.1	0.1	0.6

(a) ECL 21.58 (1.4%), 22.93 (0.2%) (ECL of arachidonate is 22.56).

(b) ECL 21.43 (1.5%), 23.13 (0.5%) (ECL of arachidonate is 22.51).

(c) ECL 21.39 (0.8%), (ECL of arachidonate is 22.22).

TABLE 2.6

Prepared methyl arachidonate after heating :-

Esters	Sample after heating	Adduct	Mother liquor	Ag^+1	Ag^+2	Ag^+1 Hydro-genation
16:0						0.5
18:0						10.5
20:0						85.5
22:0						1.5
16:1				0.6		
18:1		0.1		0.4		
18:2 (n-6)	0.8	1.0	2.8	11.0	1.7	
18:3 (n-6)	2.9	3.0	0.8	7.5	1.1	
18:3 (n-3)	3.4	3.8	1.6	6.5	1.8	
20:3 (n-6)	4.2	4.5	2.3	11.9	1.6	
20:4 (n-6)	82.2	84.0	59.6	52.6	90.0	
20:5 (n-3)	1.0	0.5	3.1	1.7	0.6	
22:4 (n-6)	1.7	1.6	8.5	1.4	1.1	
22:5 (n-3)	0.4	0.2	1.2			
22:6 (n-3)	1.4	0.2	13.7			
Others	2.0	1.1	(a) 6.4	(b) 6.4	(c) 2.1	2.0

(a) ECL 23.31 (2.7%) and 24.04 (1.8%) (ECL of arachidonate is 22.17).

(b) ECL 23.75 (5.9%) (ECL of arachidonate is 22.23).

(c) ECL 23.7 (0.7%) (ECL of arachidonate is 22.76).

B–Spectroscopic properties :-

Samples of methyl arachidonate purchased from a fatty acid supplier and ester isolated in this investigation from pig liver lipids were examined by a range of spectroscopic procedures to check the purity of the samples and to confirm that the methyl arachidonate (20:4; 5c, 8c, 11c, 14c) had been isolated.

(a) Infra-red spectra :- The main reason for examining infra-red spectra was to confirm the absence of trans unsaturation. Non-conjugated trans double bonds display characteristic absorption at 968 cm^{-1} but there was no sign of any absorption band at this position in any of the samples examined, otherwise the spectra show bands as expected at 710-715 cm^{-1} ($-(CH_2)_x-$); 1155-1244 cm^{-1} (CO_2Me); 1435-1455 cm^{-1} (C-H, bending vibration); 1740 cm^{-1} (C=O, stretching vibration); 2850-3010 cm^{-1} (C-H, stretching vibration).

(b) Ultra-violet spectra :- UV spectra are used mainly by lipid chemists to detect conjugated unsaturation. All the samples examined had a strong band at 212 nm (lg ε_{max} = 4.107). Some samples had an inflexion on the side of this peak at about 227 nm indicating perhaps some diene conjugation. This was not present in all samples and is probably the result of autoxidation which will furnish unsaturated hydroperoxides with diene conjugation.

(c) ^1H n.m.r. spectrum :- The spectrum of our 98% methyl arachidonate is satisfactorily accounted for in term of the deshielding effect of the ester function ($COOCH_3$) and the several double bonds [10]. The following signals were observed :

δ	Multiplicity	Number of protons	Assignment
0.90	triplet	3H	$-CH_3$
1.3 - 1.4	multiplet	6H	$-CH_2-$(C-17 to C-19)
1.6 - 1.8	multiplet	2H	$-CH_2-CH_2-CO_2CH_3$(C-3)
2.1 - 2.3	multiplet	6H	$-CH=CH-\underline{CH_2}-\underline{CH_2}COOCH_3$
2.83	multiplet	6H	$=CH-\underline{CH_2}-CH=$
3.68	singlet	3H	$-COOC\underline{H_3}$
5.4	triplet	8H	$-\underline{CH}=\underline{CH}-$

(d) ^{13}C n. m. r. spectra :- The ^{13}C spectrum of methyl arachidonate has already been analysed by Gunstone, Pollard and Scrimgeour [11]. Our spectrum accords well with that reported as indicated by the following comparison :-

TABLE 2.7

Carbon atom number	Gunstone et al	Present investigation
$-OCH_3$	51.40	51.39
C-1	?	173.94
2	33.50	33.50
3	24.89	24.89
4	26.65	26.65
5	128.96	128.98
6	128.96	128.98
7	25.71	25.72
8	128.25	128.26
9	128.25	128.26
10	25.71	25.72

TABLE 2.7 (continued)

Carbon atom number	Gunstone et al	Present investigation
11	127.97	127.95
12	128.65	128.64
13	25.71	25.72
14	127.63	127.66
15	130.51	130.50
16	27.29	27.30
17	29.39	29.40
18	31.59	31.61
19	22.61	22.63
20	14.05	14.06

2.4 Recommended procedure :-

The separation procedures and results set out in the preceding sections lead to the following conclusions :-

(a) The wet pig liver available to us gave about 4% of its weight as methyl esters (exluding cholesterol). These contained 9-10% of methyl arachidonate. The pig liver is a convenient, but not a very rich source of arachidonic acid and a fair amount of effort must be expended in isolating the mixed esters. A richer arachidonic acid-containing lipid is therefore desirable. In a limited investigation egg yolk furnished 7.4% of its total weight as methyl esters (exluding cholesterol), with an arachidonic acid/ester content of only 2.9%.

(b) Samples of methyl arachidonate of 98% purity are obtained by an appropriate combination of urea fractionation, silver ion chromatography, and distillation. By distillation it is possible to obtain a C_{20} concentrate containing about 25% of methyl arachidonate. This is obtained after removal of the relatively large amounts of C_{16} and C_{18} esters. As a consequence of this the arachidonate is heated to about 270°C for several hours. There is no evidence, however, of extensive decomposition or modification of the tetraene ester. Either this does not happen or the artefacts are easily removed in the subsequent purification. It is desirable to increase the efficiency of this separation, to increase the through put of the equipment, and to minimise the time of heating. These aims may not be mutually compatible and further study is required to optimise the distillation procedure.

(c) Urea fractionation proved to be a useful way of separating saturated and monoene esters as adduct from arachidonate and other polyene esters which remained in the liquor. The efficiency of this separation depends on the mixture being handled and perhaps on unknown factors which are not always adequately controlled. Three separations are recorded in Schemes 2.1 and 2.2. A 10% arachidonate fraction was divided into 27% and 1% fractions, a 25% fraction furnished 51% and 16% products and the 16% fraction gave 53% and 6% products. Each separation must be monitored and adducts recycled if they contain too much arachidonate. The procedure is simple to operate - even on a large scale and is inexpensive.

(d) Silver ion chromatography was the best method of finally upgrading the methyl arachidonate though the process may have to be repeated to get high grade products (98% and better). The high cost of

the silver nitrate must however be noted. Its cost has risen in the recent past thus : £61 (1977), £68 (1978), £91 (1979) and £159 (1980) per kilo (exluding VAT - formerly 8% but now 15%). In one of our experiments 100g of silver nitrate was used to obtain 1g of arachidonate (84%) purity (Scheme 2. 1) while in another experiment this was reduced to 20g for 81% ester (Scheme 2. 2). Even though pure methyl arachidonate commands a high price - about £36/g of 99% purity - it is clearly desirable to use the smallest possible amount of silver nitrate.

We cannot claim to have optimised these procedures but if pure methyl arachidonate has to be isolated from pig liver we recommend the following :

(i) extraction of total lipid from wet liver.

(ii) preparation of methyl esters from the lipid extract by trans-esterification.

(iii) removal of cholesterol and ether polar compounds from the methyl esters.

(iv) fractional distillation to concentrate C_{20} esters.

(v) urea fractionation to raise the concentration of arachidonate to at least 50%.

(vi) repeat Ag^{+} chromatography to get pure arachidonate.

On a laboratory scale 2kg of wet liver can provide 4g of pure arachidonate and this takes about seven man-days of work. The major difficulty in isolating large amounts of methyl arachidonate would be the inconvenience of the early stages of the separation involving very large amounts of wet liver of relatively low lipid content and large amounts of methyl ester containing only 9-10% arachidonate.

It might be helpful to use the same pig liver ester as a source of 20:5 and 22:6 and to reduce the overall costs. These two esters are present at the level of 6% and 7% in the liver ester freed from cholesterol and are presently sold at about £36 per gram each.

3. EXPERIMENTAL

Solvents : solvents of reagent grade were redistilled before use.

3.1 Analytical procedures :-

(a) Thin layer chromatography

Analytical T. L. C. was carried on glass plates (5 x 20cm or 20 x 20cm) coated with silica gel G (0. 25mm thickness). After their preparation the T. L. C. plates were dried at room temperature for 0. 5 hr then activated at 110-120°C for 2 hrs and kept in a drying cabinet containing silica.

Mixtures of petroleum (bp 40-60°C) and diethyl ether were normally used as developing solvents for T. L. C. Abbreviations such as PE20 indicate mixtures of petroleum and diethyl ether in a ratio of 80:20 by volume.

The components on analytical T. L. C. plates were detected as black spots after spraying with an ethanolic solution of phosphomolybdic acid 10% (v/v) and heating at 110-120°C.

(b) Gas liquid chromatography :-

G. L. C. analysis was carried out on a Pye series 105 chromtograph equipped with a flame ionization detector. The glass column (1. 5m long, 6mm external diameter), packed with 10% SP 2340 coated on chromosorb W AW (100-120 mesh), was used at 189-190°C. Nitrogen at a flow rate of 50ml min^{-1} was the carrier gas.

Quantitation of the peaks was based on peak height x retention distance and compositions of mixtures are reported as weight percentages. Saturated straight chain methyl esters (C_{14} to C_{24}) were used as external standards. ECL and percentage areas were calculated by computer. The variation of ECL values with aging of the column was cured by repacking the upper part of the column with fresh stationary phase, holding the column at 210°C for three hours, and then injecting it with hexamethyldisilazane.

(c) UV spectroscopy :-

UV spectra were recorded on a UNICAM SP 800 B spectrophotometer. Samples were run in spectroscopic grade cyclohexane solution using silica quartz cells (1cm wide).

(d) IR spectroscopy :-

IR spectra were recorded on a Perkin Elmer 257 grating spectrophotometer. The samples were run as thin films between sodium chloride discs. Absorption frequencies are reported in cm^{-1}.

(e) ^{1}H nmr spectra :-

^{1}H nmr spectra were recorded at 100 MHz on a Varian HA 100 instrument using deuterochloroform as solvent and 5% tetramethylsilane as internal standard. Chemical shift values are given in ppm downfield from tetramethylsilane ($\delta = 0$).

(f) ^{13}C nmr spectra :-

^{13}C nmr spectra was recorded with a Varian CFT-20 spectrometer in the Fourier transform mode of 20 MHz, with proton noise decoupling (1 KHz band width). The samples were run in deuterochloroform solution with tetramethylsilane (3% v/v) as internal standard.

(g) Hydrogenation :-

Methyl esters, methanol (1ml/1g), and palladium on charcoal were placed in a round bottomed flask, this was connected via a two-way tap to a rubber balloon filled with hydrogen. The flask was alternatively evacuated and flushed with hydrogen several times to remove any air, and finally opened to the hydrogen atmosphere. The reaction mixture was then stirred vigorously overnight with a magnet. The solution was filtered and the solvent was removed to recover the hydrogenated ester wich was then examined by gas chromatography.

3. 2 Isolation and separation procedures :-

(a) Extraction of lipid from wet liver :-

Each 100g of the tissue was homogenised for two minutes in a blender with a mixture of chloroform (100ml) and methanol (200ml). The mixture was filtered through a sintered glass funnel under water pressure and the residue rehomogenised with chloroform (100ml) and refiltered. The combined filtrates were washed with a solution of (0 . 88%) of potassium chloride in distilled water. This moved the non lipid contaminants into the upper aqueous phase. The lower lipid-containing phase was filtered and the solvent removed on a rotary film evaporator.

This procedure is based on the assumption that pig liver tissue contains about 80% of water. It is important that the final ratio of chloroform:methanol:water be as close as to 2:2:1. 8 (by volume).

(b) Transesterification and isolation of methyl ester :-

The extracted lipid was transesterified by refluxing for 2 hrs with a 2% solution of concentrated sulphuric acid in methanol (5ml/g lipid) and

methylene chloride (2. 5ml/g lipid). The solution was cooled, brine (5%) added, and the methyl ester extracted with petroleum ether (bp 40-60°C) and dried over sodium sulphate. This material is kept at -20°C to avoid autoxidation. Artificial antioxidants were not added lest they interfere with further chromatographic analysis.

The crude esters were cleaned up using a column (5 x 65cm) packed with silica (sorbsil 10g/g of ester). Elution with PE5 gave first some non-polar components (hydrocarbons) and then the methyl esters. Fractions showing only a spot ester were combined. Most of the cholesterol and the polar lipids were left in the column. The recovery of ester is about 80% of crude ester.

(c) Urea fractionation :-

The methyl esters (xg) were crystallised from methanol (8 x ml) at 0°C overnight. The precipitate was filtered and washed with cold methanol saturated with urea. Methanol was removed from the mother liquor on a rotary film evaporator. The residue was diluted with water, acidified by hydrochloric acid and the ester was extracted twice with petroleum ether. The extract, washed with distilled water and dried over sodium sulphate, gave the ester from the mother liquor.

The urea adduct was dissolved in water and extracted as described above to furnish the urea adduct esters.

(d) Argentation chromatography :-

Sorbsil (100g/g ester) and an aqueous solution of silver nitrate (10g/g ester) were mixed thoroughly. It was then activated overnight at 110°C and packed into the chromatographic column (5 x 65cm) wrapped

in aluminium foil to exclude light. The eluting solvents were PE10. PE20 and PE20 containing increasing proportions of methanol (0. 5ml, 1ml, 1. 5ml) per litre. The progress of the separation was monitored by Ag^+ T. L. C.

T. L. C. plates (5 x 20cm or 20 x 20cm) were coated with silica gel G containing silver nitrate (10%). The plates were activated at 110°C for 2 hrs and stored in desiccator protected from light. A mixture of methanol, diethyl ether and petroleum ether (2:18:80) was used as developing solvent. After development the plates were dried in a gentle stream of nitrogen and sprayed with an ethanolic solution of 2', 7'-dichlorofluorescein (0. 2%). The separated esters appeared as yellow bands under UV light. The bands were scraped off and the ester extracted with chloroform : methanol (9:1).

(e) Fractional distillation :-

The fractional distillation unit consisted of a two-necked round bottomed flask fitted to a column (2 x 60cm) packed with Fenske rings to within 5cm of its top. The column was surrounded by an electrically heated jacket and connected via a stillhead and a water-cooled condenser to a multiple receiver. The distillation unit was connected to an oil pump through an acetone cardice trap. The equipment had thermometers fitted to the pot, the jacket and the stillhead.

Methyl esters were placed in the two-necked round bottomed flask and kept under vacuum at room temperature for a while to get rid of volatile materials. The flask was then heated gradually until distillation took place. Boiling at about 135° but the temperature had to be raised further before a distillate was observed at the top of the column. The

column temperature was kept a few degrees above the distillation temperature (and was usually about 40° below the pot temperature). The reflux ratio was kept at about 1:5. In a typical distillation fractions were collected at 136-150°, 151-164°, 165-176° and 177-180° at a pressure between 0. 5 and 1. 0mm of mercury.

3. 3 Isolation of methyl arachidonate

A - Wet pig liver (995g) was extracted by chloroform-methanol to furnish (65. 9g) which was transesterified using 2% methanolic sulphuric acid (330ml) and methylene chloride (165ml) by refluxing for 2 hrs. The methyl ester was extracted with petroleum ether (3 x 600ml). The crude ester (48. 5g) was freed from cholesterol by chromatographing in a column (5 x 65cm) packed with sorbsil silica (480g). The column was eluted with PE5 and monitored by using T. L. C. plates (0. 25ml thick-developed with PE20). The recovered esters (37. 5g, 9. 6% methy arachidonate) were submitted to urea fractionation using a quantity of urea equivalent to four times the amount of saturated, monoene, and diene esters. These comprise about 70% of the total methyl esters. Accordingly 100g of urea was dissolved in methanol (200ml) with a small amount of ethanol. After crystallisation this furnished a mother liquor (9. 2g, 26. 61% methyl arachidonate) and an adduct (14. 5g, 1. 0% methyl arachidonate).

The bulk of the mother liquor (7. 8g) in two portions was further fractionated by argentation chromatography. One portion (4. 2g) was eluted from a column (5 x 65cm) packed with sorbsil silica (400g) impregnated with silver nitrate (40g) with PE10, PE20 and PE20 containing increasing amounts of methanol. The elution was monitored by Ag^+ T. L. C. The second portion (3. 6g) was fractionated by the same procedure and

the matching fractions from the two columns were combined to form products A, B and C (Scheme 2. 1). Fractions A (1. 85g, 66% methyl arachidonate), the richest in arachidonate, was applied to 10 plates of Ag^+ T. L. C. (1mm thick) and developed with methanol:ether:petroleum (2:18:80). This furnished two useful fractions A_1 (0. 85g, 84% methyl arachidonate) and A_2 (0. 03g, 76% methyl arachidonate). Full G. L. C. analysis were given in Table 2. 1.

B - Wet pig liver (1800g), extracted with chloroform and methanol, gave lipid (86g). Treated with 2% methanolic sulphuric acid (430ml) and methylene chloride (215ml) this furnished crude ester (72g, 11. 4% methyl arachidonate). The crude ester was separated into four fractions by fractional distillation (for details see Table 3. 1). The combined fractions 3 and 4 (18. 6g, 25% methyl arachidonate) were freed from cholesterol by eluting from silica (200g) in a column (5 x 65cm) with PE5. The resulting esters (17. 5g) were crystallised from urea and methanol (120ml) plus a small amount of ethanol to give a mother liquor (2. 5g, 51% methyl arachidonate) and an adduct (13. 2g, 15. 8% arachidonate). The adduct was recrystallised from urea and methanol to give a second mother liquor (2. 17g, 53% arachidonate). The G. L. C. analyses of these compounds are given in Table 3. 2.

The combined mother liquors (4. 7g, 51. 8% arachidonate) were fractionated by argentation chromatography. A column (5 x 65cm) packed with sorbsil (500g) impregnated with silver nitrate (50g), was eluted with PE10, PE20 and PE20 containing increasing amounts of methanol. The column was monitored by Ag^+ T. L. C. and G. L. C. analysis. Three main fractions were collected: A (1. 1g, 2. 1%), B (2. 5g, 81%) and C (1. 0g, 7. 8%) see Scheme 2. 2.

'ABLE 3.1

Distillation fractions	Total	1	2	3	4	Residue
Wt (g)	72	6.3	12.1	12.0	7.0	34
Distillation temp		136-150°C	151-164°C	165-176°C	177-186°C	
Component acids						
16:0	15.7	71.0	16.3			
16:1	1.2	6.5	1.1			
18:0	23.1	3.2	25.4	26.6	12.3	0.6
18:1	17.0	7.5	26.7	22.9	5.8	0.9
18:2 (n-6)	16.1	7.5	25.2	19.2	4.4	2.2
18:3 (n-6)	-	0.2	0.4	0.4	1.1	0.9
18:3 (n-3)	1.4	0.5	1.6	1.9	0.9	2.2
20:3 (n-6)	0.7		0.2	1.2	2.6	2.2
20:4 (n-6)	11.4		2.0	19.1	34.5	4.3
20:5 (n-3)	2.2		0.3	3.8	7.3	10.4
22:4 (n-6)	0.2			0.2	1.5	10.5
22:5 (n-3)	2.5			1.1	9.5	14.0
22:6 (n-3)	6.3			2.8	16.5	33.0
Others	2.2	3.6	0.8	0.8	3.6	18.8
Component acids by chain length (%)						
C_{16}	17.0	77.5	17.3			
C_{18}	58.5	18.9	79.2	71.0	24.1	
C_{20}	14.3			24.1	45.3	16.9
C_{22}	8.9				27.4	57.5
Others	1.3	3.6	3.5	4.9	3.2	25.6

These results refer to the esters submitted to distillation, the four distilled fractions, and the residue.

A further batch of pig liver (1890g) wet weight was treated in a similar way. It gave lipid (107g), crude ester (92g) and ester free of cholesterol (77g). This gave three fractions when distilled. The main fraction (40. 8g, 12. 5% methyl arachidonate), submitted to urea fractionation followed by silver ion chromatography, gave methyl arachidonate of 87% (1. 3g) and 96. 5% (0. 9g) purity.

These were combined with fraction B (2. 5g, 81%) from a previous separation (Scheme 2. 2) and resubmitted to silver ion chromatography. For this a column (5 x 65cm) was packed with sorbsil (500g) impregnated with silver nitrate (50g). The combined fractions (4. 6g) dissolved in a small amount of petroleum ether, were adsorbed on the top of the column in a narrow band. The material was slowly eluted using PE10, PE20 and PE20 containing increasing proportions of methanol. The column was monitored by Ag^{+} T. L. C. and G. L. C. analysis. Appropriate fractions were combined to give 0. 1g (24. 8%), 0. 63g (72. 4%) and 3. 5g (98. 1%).

TABLE 3.2

Esters	Mother liquor	Adduct	Mother liquor	Adduct
Wt (g)	2.5	13.2	2.2	9.9
16:0				
16:1				
18:0		33.5		42.2
18:1		21.9	2.3	26.6
18:2 (n-6)	4.1	16.9	15.7	16.7
18:3 (n-6)	0.9	0.2	0.4	0.3
18:3 (n-3)	0.4	1.8	1.8	1.8
20:3 (n-6)	1.1	1.8	3.1	1.5
20:4 (n-6)	51.1	15.8	53.0	6.1
20:5 (n-3)	14.4	2.3	9.2	0.6
22:4 (n-6)	0.9	0.4	0.7	0.3
22:5 (n-3)	2.3	2.5	5.6	1.5
22:6 (n-3)	23.9	1.7	7.9	0.3
Others	0.9	1.2	0.3	2.1

PART TWO

EXAMINATION OF RAPESEED OIL FOR THE PRESENCE OF OXYGENATED FATTY ACIDS

1. Introduction

1.1 Fatty acid composition of rapeseed oil :-

Rapeseed oil was reported to contain at least 15 fatty acids at a level of 0.5% or greater ranging in chain-length from C_{16} to C_{24}. If fatty acids at a level of below 0.5% is included the total number in certain varieties of rapeseed amount to 50 different fatty acids [12]. In a recent study [13] a number of minor unsaturated fatty acids have been isolated at a level of less than 0.1% from a low erucic variety.

In general rapeseed oils, including the new low erucic cultivars, contain saturated acids (3-7%), dienoic acids (11-13% mainly linoleic) and trienoic acids (5-15% mainly linolenic) in addition to the large amount of monoene acids.

Traditionally rapeseed oil contained a high proportion (about 48%) of erucic acid 22:1, and lesser amounts of 20:1 (about 10%) and 18:1 (about 13%). The new low erucic cultivars have higher levels of oleic (about 70%) which largely replaces the higher monoene acids: eicosenoic and erucic. That is why rapeseed can no longer be defined in the basis of fatty acid composition. According to Ackman [14] most of the monoenoic acids are of (n-9) type but a small proportion of them are (n-7) type.

1.2 Nutritional role of rapeseed oil :-

Recent nutritional and pathological studies [15] have shown the

undesirability of consuming oils and hydrogenated fats which contain high proportions of longer chain fatty acids, especially C_{22} monoenic acid, erucic acid. In experimental animals, such as the male rat, the feeding of rapeseed oil is reported [15] to give rise to cardiomyopathy. This was initially attributed to erucic acid but other oils and newer varieties of rapeseed oil giving oils low in erucic acid have recently shown similar if milder cardiomypathies.

However, it is generally agreed that partial hydrogenation to an iodine value of 77 reduces the cardiopathogenicity of rapeseed oil [16]. In view of uncertainty as to what constituent within rapeseed oil produces this undesirable effect it was suggested to us that it would be worthwhile to re-examine rapeseed oil to see if it contains furanoid or other types of oxygenated acids.

1.3 Methods of isolation of oxygenated fatty acids from vegetable oils :-

The presence of small amounts of oxygenated fatty acids in vegetable oils after long storage has been widely observed [17 to 22].

The main methods of isolation involve chromatographic procedures to concentrate oxygenated compounds in the polar fraction. Further separation according to number of double bonds can be achieved by argentation chromatography.

Furanoid acids are best concentrated by urea fractionation when such acids remain in the mother liquor together with polyunsaturated, other cyclic, and branched-chain esters. Further separation is possible by argentation chromatography [23].

This part of the thesis describes an attempt to see if rapeseed oil contains any unusual oxygenated fatty acids even at low concentration.

2. RESULTS AND DISCUSSION

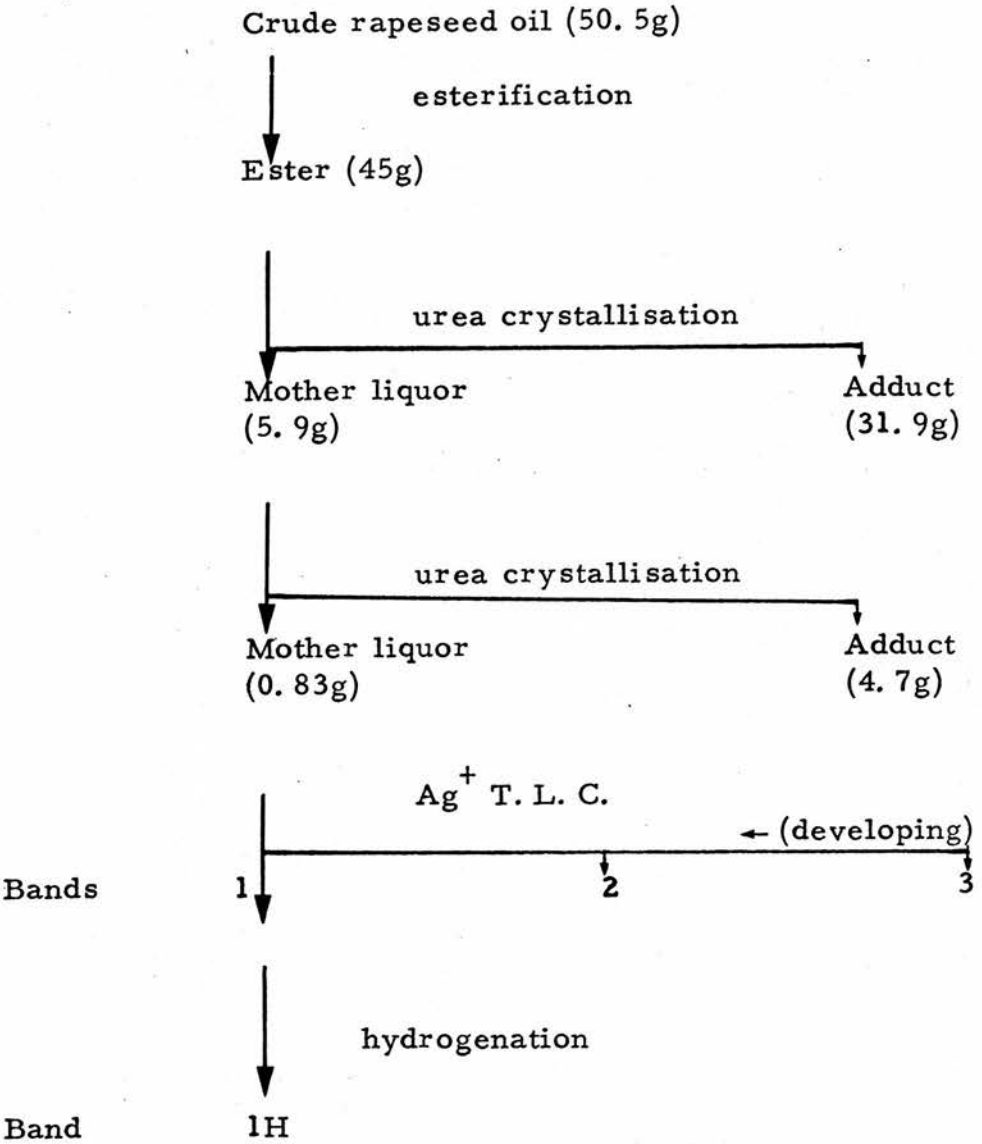
2.1 Examination of rapeseed oil for the presence of furanoid acids :-

The experimental procedure is summarised in Scheme 2.1. Crude rapeseed oil (50.5g) was transesterified with sodium methoxide in methanol and the resulting methyl esters (45g, see Table 2.1 for fatty acid composition) were crystallised from methanol in the presence of urea. Saturated and monounsaturated esters concentrated in the adduct (32g Table 2.1) and the mother liquor (5.9g Table 2.1) was mainly diene and triene esters (93%). It was however expected that any furanoid acids would also be present in the mother liquor [23].

A sample of the mother liquor submitted to preparative Ag^+ T.L.C. furnished bands enriched in 18:3 (33%) and 18:2 (100%) along with a small band containing C_{18} (23%), C_{20} (9%) and C_{22} (57%) monoenes. In order to remove these small amounts of monoenes and saturated esters from the mother liquor it was again treated with urea and methanol. This furnished a mother liquor (0.83g) containing 18:1 (2%) and saturated esters (0.6%) along with 18:2 (34%) and 18:3 (59%) esters. This fraction was submitted to Ag^+ T.L.C. A sample of furanoid esters (available from studies of fish oils) which migrate between saturated and monoene esters was run as a standard along the edge of the plates. Silica from those parts of the plates which would contain furanoid esters were eluted with petroleum ether. This extract was further examined by hydrogenation. It gave mainly the corresponding n-saturated acids (see Table 2.1). ECL (23.2) was noticed repeatedly in bands one, two, three and the hydrogenated product, which was not present in the original sample so significantly. There was no evidence of any furanoid esters.

TABLE 2.1

Compound		Total	Adduct	Mother liquor	Adduct	Mother liquor	M. L $Ag^{+}1$	M. L $Ag^{+}2$	M. L $Ag^{+}3$	M. L $Ag^{+}1$, H
Wt (g)		45	32	5.9	4.7	0.83				
ECL	Identification	%	%	%	%	%	%	%	%	%
15.9	16:0	2.2	2.6	1.8						24.4
16.8	16:1	0.1					4.3			
17.9	18:0	0.7	0.8							40.7
	16:2			1.1		0.6				
18.6	18:1		14.6	4.1	0.8		6.0			
	16:3	13.2				2.5			4.5	
19.3	18:2	12.5	7.2	45.4	45.5	33.8	30.0	92.9	12.0	
19.9	18:3 (n-6)	0.6								
	20:0									8.3
20.4	18:3 (n-3)					58.6			62.5	
	20:1	15.8	11.4	47.5	52.6		23.1			
21.3	20:3	0.2								
22.0		0.4								6.4
22.5	22:1	52.5	60.5		0.3		7.8			
23.2	22:2	0.8				1.2	25.5	5.9	14.3	20.2
24.4	24:1	0.8	0.9							
Others		0.2	2.0	0.1	0.8	3.3	3.3	1.2	6.7	0.4



Scheme 2. 1 : Examination of rapeseed oil for the presence of furanoid acid/esters.

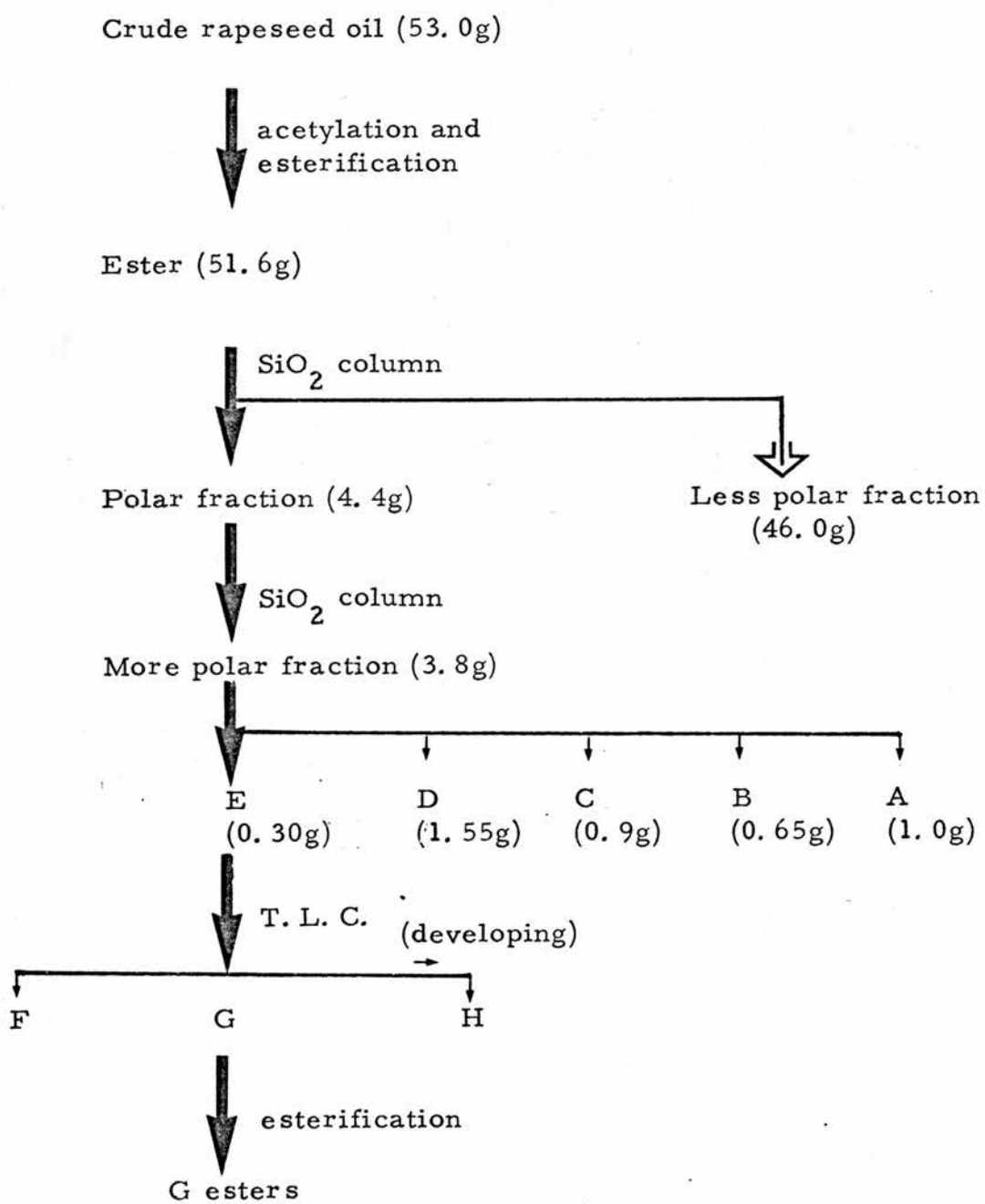
2. 2 Examination of rapeseed oil for the presence of hydroxy and epoxy acids :-

Crude rapeseed oil (53g, Scheme 2. 2) was refluxed with acetic acid and then transesterified with methanolic sulphuric acid. This should convert any epoxy compound into the corresponding dihydroxy ester. The resulting esters (51. 6g, Table 2. 2) were then chromatographed on silica to concentrate the late-running polar products. The less polar esters were eluted with PE5 and this was followed by PE20 to elute polar materials. The column was monitored by T. L. C. developed in PE20 solvent. This separation furnished polar (4. 4g) and less polar (46g) fractions. G. L. C. analysis of the polar fraction at 189°C before and after treating with hexamethyldisilazane and trimethylchlorosilane (TMS) in pyridine did not show any significant response.

The bulk of the polar fraction (3. 8g) was chromatographed again on a column of sorbsil silica and eluted with PE5, PE10, PE20 and PE35 yielding fractions A (1. 0g), B (0. 65g), C (0. 19g), D (1. 55g) and E (0. 30g). G. L. C. analysis at 254°C before and after reaction with hexamethyldisilazane and trimethylchlorosilane did not give any unusual results except possibly for fraction E. This fraction was therefore submitted to T. L. C. for further purification. Three bands were obtained. Bands F and H (Scheme 2. 2) did not seem to be of interest. Information on band G is given in Table 2. 2.

On spectroscopic examination it became apparent that G was not a methyl ester (no n. m. r. signal at $\delta = 3.6$). Instead there was a signal at $\delta = 4.1$-4. 2 suggesting the presence of a partial glyceride which must have resulted from incomplete transesterification. When this material was treated with methanolic sulphuric acid it produced a mixture of conventional

methyl esters (Table 2. 2). We thus found no evidence of hydroxy or epoxy esters in this sample of rapeseed oil.



Scheme 2. 2 : Examination of rapeseed oil for the presence of epoxy and hydroxy acids/esters.

TABLE 2.2

Total		Polar fraction*		Fraction E*		E(TMS)*		G(TMS)*		G(Ester)	
ECL	%	ECL	%	ECL	%	ECL	%	ECL	%	ECL	%
15.8	2.1			15.5	5.6					16.0	5.2
16.7	0.1										
17.7	0.7										
18.4	12.8	18.6	1.9							18.3	35.7
19.3	12.6			19.5	7.0					18.7	19.6
19.8	0.3										
20.3	15.7	20.0	4.3							20.0	70.9
21.3	0.1	21.3	0.5								
22.1	0.3			22.1	4.9					22.0	32.3
22.5	53.0										
23.1	0.7	22.9	1.5	22.9	2.2	23.7	9.8	22.2	7.0		
24.5	0.9			24.7	6.8			24.8	77.0		
		25.6	37.8	26.6	4.0	26.5	76.5				
				27.5	3.1						
		30.25	54.0	30.0	66.9	31.3	13.6	29.6	15.7		

* G. L. C. analysis at temperature 254°C.

3. EXPERIMENTAL

3.1 Analytical procedures :-

(a) Thin layer chromatography :-

Analytical T. L. C. was carried on glass plates (5 x 20cm) coated with silica gel G (0. 25mm thick). The plates were dried at room temperature, activated at 110°C and kept in a drying cabinet containing silica gel.

PE20 was normally used on developing solvents for T. L. C. and the components were detected as black spots after spraying with a 10% ethanolic solution of phosphomolybdic acid and heating at 110°C.

(b) Gas liquid chromatography :-

G. L. C. analyses were conducted on a Pye series 105 chromatograph. The glass column (1. 5m long, 6mm external diameter), packed with 10% SP2340 coated on chromsorb W AW (100-120 mesh), was used at 189-190°C (unless otherwise indicated). Nitrogen at a flow rate of 50ml min^{-1} was used as carrier gas.

Quantitation of the peaks was based on peak height X retention distance and compositions of mixtures were reported as weight percentages. Saturated straight chain methyl esters (C_{16}-C_{24}) were used as external standards. ECL and percentage areas were calculated manually.

(c) IR spectroscopy :-

IR spectra were recorded on a Perkin Elmer 257 grating spectrophotometer. The samples were run on thin films between sodium chloride discs. Absorption frequencies are reported in cm^{-1}.

(d) ^{1}H n. m. r. spectroscopy :-

The n. m. r. spectra were recorded at 100 MHz on a Varian HA100

instrument using deuterochloroform and 5% tetramethylsilane as internal standard. Chemical shift values are given in ppm downfield from tetramethylsilane (δ=0).

(e) Mass spectroscopy :-

Mass spectra were recorded on an AE1, MS902 instrument. Values of m/e are expressed as a percentage of the base peak.

(f) Hydrogenation :-

Methyl esters, methanol (1ml/1g) and 10% palladium on charcoal were placed in a round bottomed flask connected to a rubber balloon filled with hydrogen. The mixture was stirred vigorously overnight with a magnet. The solution was filtered and the solvent removed to recover the hydrogenated ester.

(g) Preparation of trimethylsilyl ethers :-

A pyridine solution (1ml) of the polar ester (1mg) was shaken for 30 seconds with hexamethyldisilazane (0. 3ml) and trimethylchlorosilane (0. 1ml). The mixture was left for 5 minutes and the product was extracted with hexane after diluting with water. The solvent was removed under reduced pressure, the flask flushed with nitrogen to remove pyridine, and the residue was then examined by G. L. C.

3. 2 Isolation and separation procedures :-

(a) Transesterification :-

Crude rapeseed oil (50. 5g) dissolved in benzene (50ml), was

refluxed with sodium methoxides (0. 5M) in methanol (100ml) for 1 hr. After cooling the reaction solution, brine (5%, 400ml) and glacial acetic acid (10ml) were added to the reaction mixture which was then extracted twice with petroleum.

(b) Acetylation and transesterification :-

Crude rapeseed oil (53g) was refluxed for seven hours with glacial acetic acid (700ml). The acetic acid was then removed under reduced pressure. Water (1 litre) was added and the product extracted twice with diethyl ether. The extract, in benzene (100ml) and methanolic sulphuric acid 2% (200ml), was refluxed for 2 hours. The solution was cooled, brine (5%) added, and the methyl ester extracted with petroleum ether.

(c) Urea fractionation :-

The methyl ester (xg) was crystallised from methanol (30x ml) and urea (5x g) at 0^{o}C overnight. The precipitate was filtered and washed with cold methanol saturated with urea. After removal of methanol from the mother liquor on a rotary film evaporator, the residue was diluted with water, acidified by dilute hydrochloric acid, and the ester was extracted by petroleum ether.

The urea adduct was dissolved in water and extracted as described above to furnish the urea adduct esters.

(d) Column chromatography :-

The methyl esters were separated into polar and less polar fractions using column (5 x 65cm) packed with silica gel (sorbsil 10g/g of ester). Elution with PE5 gave the less polar fraction. More polar material was eluted with PR20 and PE35. The separation was monitored by T. L. C. using PE20 as developing solvent.

3. 3 Examination procedures for oxygenated fatty acids :-

A - Examination for furanoid acids (see Scheme 2. 1) :-

Crude rapeseed oil (50. 5g) was transesterified with sodium methoxide in methanol [acid catalysis method is not recommended because of lability of furans towards acids]. The methyl ester (45g) was crystallised twice from methanol in the presence of urea. This furnished a mother liquor (0. 83g) enriched with dienes (18:2) and trienes (18:3) which comprise about 93% of the total. This fraction was applied to Ag^+ T. L. C. plates to concentrate the furanoid esters in the front band (between saturates and monoene esters). A sample of furanoid esters was run as a standard along the edge of the plates. The furanoid edge of the plate was sprayed with phosphomolybdic acid solution, a dark brown spot developed locating the furan level. The rest of the plate was sprayed with ethanolic dichlorofluorescein to locate the late running polyene ester bands. The assumed furan band was scraped from the plate and the extract analysed by G. L. C. before and after hydrogenation. There was no evidence of furanoid esters.

B - Examination for hydroxy and epoxy esters (see Scheme 2. 2) :-

Crude rapeseed oil (53g) was refluxed with acetic acid then transesterified with methanolic sulphuric acid. The resulting methyl esters (51. 6g) were run twice through a silica column to concentrate the late running polar fractions. The polar product E (0. 3g) submitted to T. L. C. for further purification gave band G.

^{1}H n. m. r. spectroscopic examination of this band showed the following signals :-

δ	Multiplicity	Assignment
0.9	triplet	$-CH_3$
1.28 - 1.38	multiplet	$-(C\underline{H}_2)_x-$
1.9 - 2.4	multiplet	$-C\underline{H}_2-COOCH_3$ $-CH=CH-C\underline{H}_2$
2.8	multiplet	$=CH-C\underline{H}_2-CH=$
3.7 - 4.0		glyceride H
4.1 - 4.2		atoms
5.3		$-C\underline{H}=C\underline{H}-$

The IR examination of band G showed bands at 720 cm^{-1} ($-CH_2-$); 1430-1460 cm^{-1} (C-H, bending vibration); 1730 cm^{-1} (C=O, stretching vibration); 2840-2910 cm^{-1} (C-H, stretching vibration), and 3300-3450 cm^{-1} (O-H broad intermolecular hydrogen bonded).

The mass spectrum of band G as TMS ether showed the following peaks :-

54 (9.1%)*, 55 (20.5), 56 (7.5), 57 (17.1), 58 (27.3), 60 (13.6), 68 (27.3), 69 (6.8), 70 (27.1), 74 (100), 75 (11.4) 76 (31.8), 80 (4.1), 82 (11.4), 84 (9.1), 89 (5.7), 97 (5.7), 104 (13.6), 130 (10.2), 148 (8.0).

The product was identified as partial glyceride resulting presumably from incomplete transesterification. There was no evidence of hydroxy or epoxy esters in the rapeseed oil.

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* The m/e value is followed by figures in parenthesis which indicates the intensity expressed as a percentage of the base peak.

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