Dedication

I dedicate this work to my wife Anne and son Alex.
Qualitative and quantitative determination of the cis and trans fatty-acid content of fats and oils using FTIR

Volume One

by

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ABSTRACT

The aim of this work was to assess the potential of FTIR spectroscopy in the analysis of the cis and trans fatty acid contents of fats and oils.

Preliminary studies on the liquid and solution phase spectra of individual FAME’s (fatty acid methyl esters), and on mixtures of FAME’s, were conducted to establish whether spectral features enhanced by FTIR could be used to provide improved methods of determining cis and trans contents.

A number of previously unreported bands and spectral features associated with the cis and trans isolated double bond(s) were discovered. None of the trans bands were considered suitable for quantitation, however, methods of determining the cis content were developed, based on the two bands listed below. These methods were compared with current IR methods, and with a capillary GC method.

(a) The cis C=C stretching band (= 1654 cm⁻¹).

(b) An unassigned band at ≈ 913 cm⁻¹, observed only in the spectra of non-conjugated di- and tri-unsaturated FAME’s.

All the established methods as well as those developed in this work, for the quantitative analysis of FAME’s have inherent disadvantages. For example, with the capillary GC method, difficulties are encountered in resolving and identifying samples containing complex mixtures of isomers. With the IR techniques, the difficulties involve the elimination of interfering bands, and the absorbance of similar fatty acids at the same frequencies.

The application of GC-FTIR was seen as a logical extension of this project, because it offered a great potential for the separation and characterization of components of complex mixtures. Studies conducted on vapour phase spectra showed that the identification of many FAME’s could be ascertained from various band ratios. Furthermore, band ratios could be used to quantify the cis/trans content of co-eluting isomers. Different types of capillary columns were tried, and optimum conditions established where possible.
Acknowledgements

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The help of the technical staff of the Chemistry Department is gratefully acknowledged, especially Mr. Grahamn Wright, Mr. Victor Zettel, Mr. Peter Curnock and Mr. Paul Greenwood for providing technical assistance. I am grateful to Dr Jonathan Davies and Dr Trevor Howe for the assistance given with the semiempirical molecular orbital calculations. Special thanks are also due to the staff and students of the Chemistry Department for making my three year stay so enjoyable.

I also thank my wife Anne for her encouragement, patience and support during my research studies and preparation of this thesis. Dr Steven Volsen and Dr Trevor Howe must also receive my gratitude for their persistent encouragement and guidance during the preparation of this thesis.

Finally, I would like to express my gratitude to Professor John Jones and the Ministry of Agriculture, Fisheries and Food for providing me with the opportunity to undertake the work reported in this thesis.
Declaration

The material presented in this Ph.D. thesis has not been submitted previously for any other degree or qualification and all work documented in this thesis is entirely my own. All data generated by other workers, collaborative investigations and technical assistance has been acknowledged in detail in the relevant sections of this thesis.

Format

Purly for ease of presentation, this thesis has been presented in two separate volumes. All figures and plots referred to in the text of Volume One of this thesis are presented in the same order in Volume Two.
INDEX

Title page ii
Abstract iii
Acknowledgements iv
Declaration v
Format v

CHAPTER ONE - General Introduction 1

1.1 INTRODUCTION 1
1.1.1 Triglycerides 1
1.1.2 Fatty acids 2
1.1.3 Nomenclature (shorthand designation) 4
1.1.4 Physical properties 4
1.1.5 Hydrogenation 5
1.1.6 Consumption of trans fatty acids 7
1.1.7 Biological effects of trans fatty acids 8
1.1.8 Analysis of trans fatty acids 9
1.1.9 Objectives of present work 12

1.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY 12

CHAPTER TWO - Liquid and Solution Phase Spectral Study of Fatty Acid Methyl Esters 15

2.1 INTRODUCTION 15
2.2 REVIEW ON INFRARED SPECTROSCOPY OF LIPID PRODUCTS

2.2.1 Saturated FAME's
2.2.2 Cis FAME's
2.2.3 Trans FAME's
2.2.4 Conjugated unsaturated FAME's

2.3 EXPERIMENTAL

2.3.1 Perkin Elmer 1750 FTIR system
2.3.2 FTIR scanning parameters
2.3.3 Source of purchased FAME's
2.3.4 Capillary GC analysis of FAME's
2.3.5 Storage of FAME's
2.3.6 Preparation of deuterated methyl elaidate
2.3.7 Preparation of methyl-d3-stearate
2.3.8 Spectral enhancement (deconvolution)
2.3.9 Preparation of linoleic acid
2.3.10 Preparation of mixed isomers of methyl linoleate
2.3.11 Semiempirical molecular orbital calculations

2.4 RESULTS AND DISCUSSION

2.4.1 Comparison of the "normal" and enhanced spectra of various unsaturated cis and trans FAME's
2.4.2 Region 1 - 4000 to 2600 cm⁻¹
2.4.2.1 Spectral differences in region 1 between different cis mono-unsaturated and saturated FAME's
2.4.2.2 Spectral differences in region 1 between various poly-unsaturated FAME's
2.4.2.3 Spectral differences in region 1 between various trans unsaturated FAME's
2.4.2.4 Spectral differences in region 1 between methyl linoleate and the various conjugated isomer fractions
2.4.3 Region 2 - 2600 to 1600 cm⁻¹
2.4.4 Region 3 - 1600 to 1000 cm⁻¹
2.4.5 Region 4 - 1000 to 400 cm⁻¹
CHAPTER THREE - FTIR Quantitative Studies of Cis and Trans FAME's

3.1 INTRODUCTION

3.2 LITERATURE REVIEW OF THE DETERMINATION OF CIS/TRANS FATTY ACIDS

3.2.1 Determination of trans fatty acids by infrared spectroscopy

3.2.2 Determination of cis FAME's by infrared spectroscopy

3.2.3 Analysis of fatty acids by gas chromatography

3.2.3.1 Packed columns

3.2.3.2 WCOT columns

3.3 EXPERIMENTAL

3.3.1 Preparation of the calibration solutions

3.3.2 Scanning conditions

3.3.3 Subtraction of solvent spectrum from the solution spectra

3.3.4 Determination of the peak height absorbance using the straight baseline correction technique

3.3.5 Determination of the peak height absorbance using the curved baseline simulated (CBS) correction technique

3.3.6 Integrated peak area as a measure of the peak absorbance

3.3.7 Smoothing and flattening of spectra

3.3.8 Determination of the extinction coefficient of cis and trans bands (using reference standard FAME's)

3.3.9 Demountable cell used

3.3.10 Methylation methods used on fats and oils

3.3.10.1 Methanolic potassium hydroxide methylation method

3.3.10.2 Tetramethyl ammonium hydroxide methylation method

3.3.11 TLC optimisation of methylation methods

3.3.12 Preparation of FAME's solutions from the samples of fats and oils
3.3.13 Determination of the cis and trans content by IR

3.4 RESULTS AND DISCUSSIONS

3.4.1 Selection of solvent for solution phase studies of FAME's and solvent component subtraction technique

3.4.2 Stability of methyl elaidate in CCl₄

3.4.3 Extinction coefficients of the trans band (baseline corrected) of various trans FAME's

3.4.4 Determination of the trans % content of model mixtures using the peak height straight baseline correction technique

3.4.5 Determination of the trans content of various samples

3.4.5.1 Fat samples - B72E and a trial fat

3.4.5.2 Repeatability of the % trans content by the capillary GC & FTIR methods and assessment of different calibration solutions

3.4.5.3 Comparison of two different two component calibration data on the determination of the % trans content of various biscuit fats

3.4.5.4 Comparison of % trans content obtained by peak height and peak area measurements

3.4.6 Cis =C-H stretching band quantitative studies

3.4.6.1 Extinction coefficients of the cis band at ≈ 3005 cm⁻¹ of various FAME's

3.4.6.2 Determination of the cis content of simulated mixtures using the absorbance of the cis =C-H band at 3005 cm⁻¹

3.4.6.3 Determination of the cis content of fats and oils using the cis =C-H stretching band absorbance

3.4.7 Cis C=C stretching bands quantitative studies

3.4.7.1 Extinction coefficient of the cis and trans C=C stretching band of various FAME's using the straight baseline correction technique

3.4.7.2 Cis content determination of model methyl oleate/methyl elaidate mixtures using the straight baseline correction technique

3.4.7.3 Cis C=C band extinction coefficient of cis poly-unsaturated FAME's using the straight baseline correction technique

3.4.7.4 Development of a curved baseline technique in the determination of the peak height of the cis C=C band

3.4.7.5 Cis C=C stretching band extinction coefficient of various FAME's using the cis curved baseline simulation technique
3.4.7.6 Determination of the cis content of model mixtures using the cis C=C band and the CBS technique

3.4.7.7 Extinction coefficient of the cis C=C stretching band of poly-unsaturated FAME's

3.4.7.8 C=C stretching bands of conjugated isomers

3.4.7.9 Determination of the cis content of fats and oils using the cis C=C band and CBS technique

3.4.7.10 Repeatability measurements of the cis content by capillary GC and FTIR

3.4.7.11 Collaborative study of biscuit fats

3.4.7.12 CBS technique: further developments

3.4.7.13 Comparison of cis C=C peak height and area CBS techniques on samples high in either trans or cis poly-unsaturated FAME's

3.4.8 Quantitation of isolated cis di- and tri-unsaturated FAME's

3.5 CONCLUSIONS

CHAPTER FOUR - GC-FTIR Study of FAME's

4.1 INTRODUCTION

4.2 LITERATURE REVIEW

4.3 EXPERIMENTAL

4.3.1 Heated gas cell

4.3.2 GC-FTIR

4.3.3 Calculation of mean noise

4.4 RESULTS AND DISCUSSIONS

4.4.1 Vapour phase spectrum of methyl elaidate

4.4.2 Preliminary GC-FTIR experiments using the DTGS detector

4.4.3 Sensitivity of GC-FTIR system using the MCT IR detector

4.4.4 Discussion of IR chromatogram

4.4.5 Vapour phase spectra of various FAME's obtained using the narrow bore capillary GC-FTIR system
4.4.6 GC-FTIR study of fat N88 using narrow bore capillary column
4.4.7 GC-FTIR investigations with wide bore column
4.4.7.1 Influence of peak broadening upon quantity of eluent in the light pipe
4.4.7.2 Optimisation of GC-FTIR system with wide bore capillary column for mixtures of five saturated FAME's
4.4.8 Absorbance bands ratios
4.4.8.1 Assessment of the standard deviation of the band ratios
4.4.8.2 Band ratio plots of saturated FAME's
4.4.8.3 Band ratio plots of unsaturated FAME's
4.4.8.4 Band ratio plots of methyl oleate/methyl elaidate mixtures produced with the wide bore column
4.4.8.5 Homogeneity of co-eluting methyl oleate and methyl elaidate peak
4.4.9 Mega bore column/GC-FTIR system
4.4.9.1 Band ratio studies produced by GC-FTIR system utilising a mega bore column
4.4.9.2 Effects of sample concentration and volume injected into the mega bore capillary GC-FTIR system upon quality of the spectra obtained
4.4.9.3 Calibration plots obtained from mixtures of methyl oleate/methyl elaidate using the mega bore column

4.5 SUMMARY/CONCLUSIONS

REFERENCES

APPENDIX I

APPENDIX II
CHAPTER ONE

General Introduction

1.1 INTRODUCTION

The work reported in this thesis was sponsored by the Ministry of Agriculture Fisheries and Food (MAFF) to assess the potential of Fourier Transform Infrared (FTIR) spectroscopy in the analysis of fats and oils. This chapter is subdivided into two sections. The first covers the reasons why this work was undertaken and includes brief descriptions of the composition, analysis and nutritional value of fats and oils. The second section covers FTIR spectroscopy, and discusses the advantages that FTIR instruments have over conventional dispersive infrared (IR) instruments. Various subjects covered in these sections will be elaborated upon in subsequent chapters.

1.1.1 Triglycerides

Triglycerides are esters of glycerol and fatty acids (Figure 1.1.1), and are generally referred to as simple lipids (Christie, 1989). They form just one category of a diverse family of naturally occurring lipophilic compounds which also include the ester waxes and phospholipids. Lipids are insoluble or sparingly soluble in water, but soluble in most organic solvents and all of them contain one or more fatty acid groupings in their molecular structure.

Triglycerides are found throughout the plant and animal kingdom and are essential to living matter. They are the principle constituents of fats (solid state) and oils (liquid state), which are laid down by plants and animals mainly as energy reserves. The large number and complexity of the natural fats and oils reflect both the mode of arrangement of the individual fatty acids which are attached to the glycerol skeleton to form specific glycerides, and the relative proportions of such glycerides which are present in the specific fat or oil.
1.1.2 Fatty acids

This investigation into glycerides stems largely from the fatty acid combination which they contain. Fatty acids are so called because they were isolated from fats (by hydrolysis) and characterised as acids by their ability to form salts and bases. Fatty acids are carboxylic acids (RCOOH) and are distinguished from one another by the character of the aliphatic R group, which can be saturated or unsaturated.

Normal saturated fatty acids have a unbranched R group and can be represented by;

\[ \text{CH}_3(\text{CH}_2)_n\text{CO}_2\text{H} \]

The systematic and trivial names, formulas, formula weights and shorthand designation of all members of the series formic to triacontanoic, are given in Table 1 (Appendix I). All of the even number (n = even) acids over the range 2-24 occur either in the free or combined state in nature, with only traces of odd numbered acids being present, this also applies to the unsaturated fatty acids. The two most abundant saturated fatty acids in plant and animal tissues are shown on the following page;

a. Palmitic acid (trivial name) \[ \text{CH}_3(\text{CH}_2)_{14}\text{CO}_2\text{H} \]

b. Stearic acid (trivial name) \[ \text{CH}_3(\text{CH}_2)_{16}\text{CO}_2\text{H} \]

Straight-chain unsaturated fatty acids are characterised by the presence of one or more double bonds. There are so many acids in this class that it is convenient to subdivide them into three groups; the mono-unsaturated which contain only one double bond, the non-conjugated (isolated) poly-unsaturated which contain more than one double bond separated by one or more methylene groups, and finally the conjugated poly-unsaturated which contain at least two conjugated double bonds. The mono-unsaturated fatty acids, also termed mono-alkenoics/mono-olefinic, encompass the largest number of known naturally occurring and synthetic unsaturated acids. Table 2 (Appendix I) shows the systematic and trivial names, formulas, formula weights and shorthand notation of various members of this series which have been studied by the author.

The large number of theoretically possible mono-ethenoic acids result not only from differences in the length of the carbon chain, but also from the occurrence of positional isomers of the carbon-
carbon double bond, and, more importantly with respect to this project, there exist two geometric isomers (Figure 1.1.2). The configuration of the naturally occurring mono-unsaturated fatty acids is nearly always cis. The main sources of trans fatty acids will be discussed in a later section. Cis mono-unsaturated fatty acids with 18 carbons or less are liquids at room temperature, trans-isomers have somewhat higher melting points. The most abundant mono-unsaturated fatty acid in tissues is cis-9-octadecenoic acid, also termed oleic acid, which has the following structure:

$$\text{CH}_3-(\text{CH}_2)_7-\text{CH}))-\text{CH}-(\text{CH}_2)_7-\text{COOH}$$

In naturally occurring fats and oils high in poly-unsaturates, the main constituents tend to be non-conjugated, with cis double bonds. Examples of some of the more important of these acids are shown in Table 3 (Appendix 1). Linoleic acid (cis-9 cis-12-octadecadienoic acid) is the most widespread fatty acid of this type and it is found in most animal and plant tissues. It is an essential fatty acid in animal diets for normal growth, reproduction and healthy development, yet it cannot be synthesised in animal tissues. The enzymes in animals are only able to insert new double bonds between an existing double bond and the carboxyl group. Linoleic acid, therefore, serves as the precursor of a family of fatty acids that is formed by desaturation and chain elongation. These compounds have profound pharmacological effects and are the subject of intensive study. Linolenic is an important intermediate in the biosynthesis of these further family of poly-unsaturated fatty acids, and is a constituent of certain seed oils. In general, poly-unsaturated fatty acids have low melting points, and are susceptible to oxidative deterioration or autoxidation.

To summarize, most of the naturally occurring saturated and unsaturated fatty acids have an even number of carbons in their carbon chain due to the manner in which they are biosynthesised (Gunstone, 1967a). The poly-unsaturated fats and oils are mainly non-conjugated, and the configuration of the double bonds is invariably in the cis position. The following broad generalisations can be made about the fatty acid composition found in animal, vegetable and fish fats and oils, which are of interest with respect to this work. In all these categories, the common fatty acids vary in chain-length from 14-22, but on occasion can span the range from 2 to 36 or even more, these though tend to occur as minor components. Fatty acids from animal fats may have one to six double bonds, although the main components are generally saturated. Vegetable oils rarely have more than three double bonds and these tend to be the main components, whereas the fatty acids of fish oils may have one to six double bonds which occur as the main components. It should be emphasized that these statements are extremely broad and that large differences in the fatty acid composition will occur within each of these categories as well as
species. The significance of the fatty acid composition of these fats and oils will become apparent in the following sections.

1.1.3 Nomenclature (shorthand designation)

The trivial and systematic names of the majority of the fatty acids studied can be found on Tables 1, 2 and 3 in Appendix I. Also included in these tables is the shorthand designation which is a useful abbreviation used to describe the structure of a fatty acid (Gunstone, 1967b). This system is widely accepted and will be used throughout this thesis. According to this system the first number (18), is the total number of carbons in the fatty acid. The second number after the colon refers to the number of double bonds (1). The symbol Δ with the number after it designates the position of the double bonds in the carbon chain starting from the carbonyl carbon. The final c or t refers to cis or trans configuration.

\[
\text{CH}_3-(\text{CH}_2)_7-\text{CH}^\text{cis}=\text{C}^0\text{H}-(\text{CH}_2)_7-\text{C}^1\text{OOH} \quad (18:1\Delta 9c)
\]

\[
\text{CH}_3-(\text{CH}_2)_4-\text{CH}^\text{cis}=\text{CH}-\text{CH}_2-\text{CH}^\text{cis}=\text{CH}-(\text{CH}_2)_4-\text{CH}^\text{trans}=\text{CH}-\text{CH}_2-\text{COOH} \\
(18:3\Delta 9c12c)
\]

1.1.4 Physical properties

The fatty acid composition has fundamental effects on the physical properties of triglycerides. In a triglyceride containing only saturated fatty acids the long chains of the fatty acid are extended in a linear fashion (Figure 1.1.4.1) as are the chains of carbons in an unsaturated fatty acid with a trans configuration (Figure 1.1.4.2). This means that in the solid state, the molecules of a triglyceride can mesh together giving rise to strong intermolecular forces, and hence a high melting point. Thus, at room temperature lipids high in saturated or trans mono-unsaturated fatty acids tend to be solids, and referred to as fats (e.g. animal fat, see section 1.1.2).
In a triglyceride containing only cis unsaturated fatty acids, there is a bend in the chain caused by the configuration of the double bond (Figure 1.1.4.3), thus molecules align poorly (including in mixtures containing saturated or trans). The net result is that cis unsaturation lowers the melting point of a triglyceride mixtures. This explains why lipid mixtures with a highly cis unsaturated fatty acid content tend to be liquids at room temperature and are referred to as oils (e.g. vegetable and fish oils, see section 1.1.2). Fatty acid structure and physical properties have been reviewed by Gunstone (1967c).

In the liquid phase the methylene carbons have free rotation (i.e. the methylene chains are totally flexible). Thus the structures shown in figures 1.1.4.1 to 1.1.4.3 are possible minimum energy conformations when surrounded by molecules of similar structure. These conformations have been verified by crystallography (saturated, trans and cis fatty acids, von Sydow,1956; Mitcham, 1973; Abrahamsson, 1962; respectively). However, the crystalline nature of fatty acids is complicated by polymorphism (Markley, 1968A). Polymorphism is particularly important to the fat and oil industry because the consistency of many products, such as lard, butter, shortening, margarine etc., is dependent on the particular polymorphic modifications that are induced in these products during processing and use.

1.1.5 Hydrogenation

One of the most important factors to be addressed by the fat and oil industry is fatty acid composition, since the levels of saturated and cis unsaturated fatty acids determine the physical state of the finished product. The finished product is modified by either blending or processing (hydrogenation) to achieve the texture desired by the consumer. This is exemplified by the traditional use in the United States and United Kingdom of lard and butter (high in saturated fats) for cooking purposes. This has led to a prejudice against the use of cheaper, equally nutritious vegetable oils, which are high in non-conjugated cis poly-unsaturated fatty acids.

Partial hydrogenation (Coenen, 1976; Patterson, 1983) of the double bonds in cheap vegetable oils such as cottonseed oil, corn oil and soyabean oil converts these liquids into solids thus having a more desirable consistency for the consumer and also improving their flavour and stability. Hydrogenation not only eliminates some of the double bonds but, more importantly, causes isomerisation of a significant proportion of the remaining cis double bonds to the
thermodynamically more stable trans configuration, resulting in the formation of variable amount of trans monoenes and cis/trans non-conjugated dienes, as in soyabean oil (Applewhite, 1981). The level and type of trans fatty acids produced during the industrial hydrogenation of oils are numerous because of the wide variety of fish and vegetable oils which are treated, and because any one oil may be subjected to any of a number of hydrogenation conditions, each yielding differing degrees of unsaturation and a different pattern of isomers.

The formation of position isomers during partial hydrogenation of oils depends upon the fatty acid composition of the oil and degree of hydrogenation (Smallbone, 1985). Partial hydrogenation of poly-unsaturates to the mono-unsaturates can lead to more than one positional isomer depending upon which double bond is reduced. Positional isomers are also produced via the formation and subsequent partial hydrogenation of conjugated fatty acids. Examples of the possible hydrogenation products of linoleic and linolenic acid are shown in Table 1.1.5.1 below;

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Hydrogenation products</th>
</tr>
</thead>
<tbody>
<tr>
<td>Linoleic acid</td>
<td>18:1Δ9&lt;sup&gt;e&lt;/sup&gt;, 18:1Δ12&lt;sup&gt;e&lt;/sup&gt;, 18:2Δ9-11&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>18:2Δ9c12c &lt;sup&gt;c&lt;/sup&gt;</td>
<td>18:2Δ10-12&lt;sup&gt;c&lt;/sup&gt;, 18:1Δ11&lt;sup&gt;d&lt;/sup&gt;, 18:1Δ10&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>Linolenic acid</td>
<td>18:1Δ9, 18:1Δ12, 18:1Δ15, 18:2Δ9,12</td>
</tr>
<tr>
<td>18:3Δ9c12c15c</td>
<td>18:2Δ9,15, 18:2Δ12,15 etc.</td>
</tr>
</tbody>
</table>

<sup>c</sup> = conjugated products; <sup>d</sup> = partial hydrogenated products of conjugated fatty acids  
<sup>e</sup> = 18:1Δ9 and 18:1Δ12 hydrogenated products can be produced by partial hydrogenation of either the starting fatty acid or the conjugated fatty acids.

Trans acids occurring in hydrogenated vegetable oils such as soyabean, rapeseed and palm oils are predominantly a mixture of C18 mono-unsaturates - elaidic and its positional isomers (18:1 trans isomers with the double bond occurring at different positions in the fatty acid chain). Smaller proportions of dienes derived from linoleic acid, e.g. 18:2c/t and 18:2t/t, are also often present. Concentrations of trans trienes derived from linolenic acid are generally very low. It should be noted that not all of the oleic acid in these vegetable oils is reduced or isomerised to the trans
isomer during hydrogenation. For example, hydrogenation of soyabean oil, a highly poly-
unsaturated oil, produces both 18:1Δ9c and 18:1Δ12c monoenes from the reduction of linoleic
acid and a broad spread of trans 18:1 monoenes centred on the 9, 10 and 11 isomers originating
from the conjugated 10-12 and 9-11 isomers of linoleic acid. In contrast, rapeseed oil, a
comparatively less poly-unsaturated oil containing a large proportion of oleic acid, when hardened
contains both the cis and trans- 18:1 monoenes predominantly in the 18:1Δ9 position
(Smallbone, 1985). The partial hydrogenation of highly unsaturated fish oils containing fatty acids
with 5 and 6 double bonds creates very broad distributions of positional isomers. This is due to
the numerous possibilities for producing positional isomers from poly-unsaturated fatty acids such
as 18:5 and the high degree of hydrogenation required.

1.1.6 Consumption of trans fatty acids

Industrially hydrogenated fish and vegetable oils are used in the manufacture of a variety of
consumer and industrial products including frying oils, margarines, shortenings and speciality
products which, in turn, may be incorporated into snacks, fried products, bakery products,
confectioneries and other foods. Of these, margarines, oils, fats and flour-based products are a
major source of dietary fat in many countries. Studies by Jackson et al. (1983) on US fat
consumption from animal and vegetable sources have shown an increased consumption of
partially hydrogenated vegetable oil, which by 1980 accounted for more than three times as much
as in the 1909-1913 period. In addition, the proportion of the total fat provided by vegetable
sources increased from 17% to 42% during this period, although animal sources still contribute a
greater proportion of the total fat in the US food supply. In consequence, trans fatty acids are a
common constituent of the Western diet due to the extensive use of hydrogenated oils in
processed food.

Trans fatty acids are also found in all fat-containing products derived from ruminants (Garton,
1977; Patton and Jensen, 1975), particularly products derived from cow's milk fat. Unlike
monogastrics, ruminants are able to produce trans fatty acids by means of the rumen flora. An
other less important source of trans fatty acid is oxidation (Frankel, 1985), which during
processing or storage of food may cause a further increase in these constituents.

Total daily intake of trans fatty acids, based on food consumption data, has been estimated by
Burt (1984) to be, on average, some six per cent of the total dietary fat (approximately 7 grams per head, per day), although some individuals may in extreme cases consume 27g or more trans fatty acids per day, and others as little as 5 grams. When high levels of trans fatty acids are consumed, a major proportion is derived from hydrogenated fish oil. At the lower level of consumption, the main sources of trans fatty acids are vegetable oils, milk and ruminant carcass fats. In the case of the average consumption (7 grams per head, per day) the source of the trans fatty acid is equally distributed between ruminant and hydrogenated oils.

"Trans Fatty Acids", a report prepared by the British Nutrition Foundation's Task Force (1987), suggested that the general trends in fat consumption, compiled from commercial information, is towards a relatively high consumption of unsaturated vegetable oils; the proportion of hydrogenated fish oil, responsible for high trans consumption, in the fats and oils incorporated into UK food, was very steady during the period 1980-1985 but then dropped from 26% in 1985 to 22% in 1986. This was attributed to two factors, the first was economic. The price of fish oils has been relatively lower than that of other edible oils, but during the latter period the price difference has eroded. The second factor is that of the consumer attitude which since the recent UK report by the Department of Health and Social Security (1984) on "Diet and Health" has shown an increased preference for products based on vegetable oils.

Although current trends indicate that the levels of trans fatty acid in the UK diet, especially those of fish origin, are likely to continue to diminish, the levels of trans fatty acids have increased dramatically since the turn of the century. This dietary change has generated interest in the health implications of increased consumption of the resultant trans fatty acids.

1.1.7 Biological effects of trans fatty acids

The biological effects of trans-isomers resulting from biohydrogenation or the industrial hydrogenation of vegetable oils have been comprehensively reviewed (FASEB, 1985; Zevenbergen, 1986), as have those of industrially hydrogenated marine oils (Barlow, 1984). The biological effects of trans fatty acids depend on the amount consumed, chain length and the number and position of the double bonds. Few studies have examined the influence of purified trans fatty acids and knowledge about their effects has been derived in the main from observations with diets containing hydrogenated fats. The interpretation of such studies have
not taken into account other variables such as the intake of saturated and essential fatty acids.

The previous paragraph explains why there are a number of conflicting reports on the effects of trans unsaturated fatty acids upon coronary heart disease, the main area of concern with respect to the health implications of trans fatty acids. Some studies (Vergroesen, 1972; McMillan, 1973) suggest that trans unsaturated fatty acids cause an elevation of plasma cholesterol, while Applewhite (1981) found no change in plasma cholesterol and in glyceride levels in men fed trans unsaturated fatty acids. Recent reviews by Kanhai (1988) and the BNF (1987) both concluded that there appeared to be no reliable evidence to suggest that trans fatty acids, of the type currently consumed within the UK and in the amounts likely to be consumed, pose a measurable risk to human health. However, in the absence of firm data, recommendations were made that the levels of trans, trans dienes fatty acids arising during the industrial hydrogenation of vegetable and marine oils be maintained at the minimum practicable levels. Currently the MAFF are considering the implementation of the COMA (1984) report on "Diet and Cardiovascular disease" which recommended the inclusion of a combined trans and saturated fatty acid content in labelling of fatty foods.

1.1.8 Analysis of trans fatty acids

The information presently required for nutritional labelling of foods does not include trans conjugated, or mono-unsaturated fatty acid contents. Should the new labelling regulations being planned by MAFF require a declaration recommended by the COMA (1984) report, analytical methods suitable for a wide range of food fats will need to be chosen. The three major techniques commonly used for the determination of these acids are IR spectroscopy, gas chromatography and argentation chromatography. Each of these will be discussed briefly; more detailed reviews are presented in later chapters.

A total of 14 methods have been found in the literature that describe the determination of trans acids by IR spectroscopy. Of these, three are official methods. All of these methods are based on the fact that isolated trans double bonds in long-chain acids, esters and glycerides have an absorption band at approximately 967 cm\(^{-1}\) arising from the carbon hydrogen (\(\equiv\text{C}-\text{H}\)) deformation about the trans double bond, which does not occur in either cis or saturated fatty acids. Measurement of this absorption band under controlled conditions is the basis for the quantitative
(spectroscopic) determination of the trans content.

The IR analysis procedures are simple, rapid and accurate, and require minimal time in sample preparation, but they give no information concerning specific structure of individual fatty acids and do not take into account possible differences in trans absorbances for fatty acids of different chain length or positional isomers, upon which few studies have been conducted. Another source of error with these methods is caused by peak overlap between the trans band and various bands occurring in the acids, triglycerides and methyl esters. This problem has been corrected for by the IUPAC (1979) method, which specifies the use of methyl esters (whose level of interference is less than that of either the acids or triglycerides) and measures samples against a blank containing methyl stearate (18:0) at the same concentration as the sample. It has been successfully employed for the determination of trans acids in many margarine oil and other food fats (Kochhar, 1984). The present author is critical of the fats and oils selected because the main components were either saturated or unsaturated methyl esters with eighteen carbons in the acid chain, thus resembling the methyl stearate blank. The question is therefore asked, whether this method would be as successful on fats and oils whose composition did not consist primarily of fatty acid methyl esters with eighteen carbons in the acid chain.

Gas chromatography (GC) is used extensively for the analysis of fatty acids and their methyl esters and can be performed with either packed or capillary columns. Most GC systems using packed columns with moderate polar polyester or cyanosilicone phases provide separation on the basis of chain length and the number of double bonds, and not according to type of double bond. Separation of cis/trans isomers is only achieved on the highly polar dicynoalkyl silicone stationary phases, OV-275 (Witting et al., 1984). On these highly polar phases the saturated fatty methyl esters elute before the trans, which are in turn eluted before the cis isomer of the same chain lengths. However, individual isomers of the cis- and trans-monoenes are not separated.

The OV-275 stationary phase is used in the AOCS official method Cd 17-85 (1985), which was developed in the United States to determine the trans and cis content of fats and oils. It appears that acceptable results can be obtained on margarine fats based on hydrogenated vegetable oils in which the major trans acid component is 18:1t, with smaller proportions of 18:2tt isomers. Such packed columns are unable to separate the complex mixtures of isomers found in hydrogenated fish oils. These fats are employed extensively in much of the world for margarine manufacture, except in the United States where hydrogenated vegetable oils are exclusively used.

The recent development of capillary column gas chromatography (CCGC) using improved
stationary phases such as Silar 10C, SP2340 and OV-275 has resulted in a number of studies (Sampugna et al., 1982; Ackman et al., 1971; Ackman et al., 1974; Quinn et al., 1978; Ojanpera, 1978; Heckers et al., 1977; Slover et al., 1979) measuring the trans content of commercially available food, mainly margarines. The trans content determined in this way has been compared with IR results.

The polar phases mentioned above were shown to separate cis and trans monoenes with the double bond in the same position, positional isomers having the same configuration/chain length and finally number of double bonds. The problems with these CGGC methods arise from the overlap of cis and trans positional isomers and unidentified peaks which effect quantification of the trans content (see Chapter 3 for further details of these problems and a review of packed and capillary GC analysis). Consequently, accurate analysis of fats containing hydrogenated fish oil is not possible and even for hydrogenated vegetable oils capillary GC cannot be relied upon to give accurate results for all types of fat due to variable spread of positional isomers. In addition, the variety of stationary phases, columns and conditions being used make it difficult for different laboratories to obtain reproducible results.

Argentation chromatography, using silver nitrate impregnated silica gel, employs the ability of unsaturated compounds to complex with silver ions. Cis double bonds complex more strongly than trans isomers so consequently they elute more slowly. The most commonly practised application of argentation chromatography for trans determination involves silver nitrate thin layer chromatography (TLC) combined with gas chromatography (GC). This technique employs preparative silver nitrate-TLC to separate the cis and trans isomers of fatty acids (as methyl esters), which are quantified by gas chromatography with an internal standard (Scholfield, 1979; Conacher, 1976; IUPAC method, 1979b; Christie, 1971). Fatty acids can be separated according to both the number and the configuration of the double bonds and sometimes, with care, according to the position of the double bonds in the alkyl chain (Morris et al. 1967).

In fats containing saturated and unsaturated fatty acids with predominantly eighteen carbons in the alkyl chain, both trans monoenes and trans dienes are easily separated. With hydrogenated fish oils, containing a range of saturated and unsaturated fatty acids of different chain lengths, problems are encountered with overlapping bands due to partial separation according to chain length. In addition, overlap of trienes with cis dienes may occur with these fats. Sebedio (1983) developed a method which alleviates these problems by using a preliminary TLC separation according to degree of unsaturation using the methyl ester-mercury acetate adduct. The resulting monoenes, dienes and trienes can then be chromatographed separately to give their trans
isomers. This method, and the classical silver nitrate-TLC/GC procedure overcome the separation problems encountered by conventional GC and permit accurate results in the right hands, but are highly exacting and unsuitable for routine analysis.

1.1.9 Objectives of present work

Both the IR and CCGC methods of measuring the trans contents of fatty acids in food have inherent disadvantages. These disadvantages could be overcome by integration of CCGC with FTIR (i.e. a GC-FTIR system), coupling the separating ability of gas chromatography with the qualitative specificity of IR spectroscopy. Such a system would allow all the components to be identified, thus accurately measuring the trans content of the fat. Any FAME's which remain unresolved could be identified if one or more of the components which overlap is known by using a computer program, to subtract the spectra of the identified component (stored in spectral library) from that of the mixture.

Continuing improvements in gas chromatography-Fourier transform infrared spectroscopy have resulted in making capillary column GC-FTIR a practical analytical tool. Primarily as a result of the development of improved light-pipes and low dead volume interfaces, useful vapour-phase IR spectra of compounds present in quantities as small as 10 to 100 ng can be obtained "on-the-fly" (Griffiths et al., 1983).

The main objective of the present work was to develop and assess a GC-FTIR system which would allow the determination of the cis and trans content of fats and oils. In addition, the liquid and solution phase spectra of individual and mixtures of FAME's, produced on the FTIR spectrometer, were examined in order to establish whether spectral features enhanced by FTIR could be used to provide improved methods of determining the cis/trans content. For example use might be made of spectral bands of low intensity or bands which overlap.

1.2 FOURIER TRANSFORM INFRARED SPECTROSCOPY

This section reviews the various stages involved in the determination of a spectrum by FTIR, the
general methodology is outlined, however, no attempt is made to provide an in-depth description of either the general IR theory or the details of the algorithms. Emphasis is placed on the advantages of FTIR over dispersive instruments and how these advantages can be applied to the problems set in the objectives.

The FTIR spectrometer is a multiplex instrument. The term multiplex comes from the communication industry, where it is used to describe systems in which many sets of information are transported simultaneously through a single channel. As the name implies all the individual transmittance measurements that go to make a spectrum (resolution elements), which are at equally spaced frequency or wavelength intervals, are measured simultaneously on the FTIR spectrometer. Thus, an entire spectrum can be recorded in the time required to observe just one resolution element by a dispersive instrument which has to record each resolution element individually. Many scans can be averaged in the time taken for a single scan on a dispersive instrument. This allows spectra of the same signal-to-noise ratio (SNR) to be measured m times faster on a FTIR spectrometer than on a monochromator, where m is the number of resolution elements in the spectrum. For equal measurement times, the SNR of spectra measured on a FTIR spectrometer is √m times greater. This advantage of Fourier transform spectroscopy was first recognised by Fellget (1958) and has become known as the Fellget advantage. A schematic diagram of the essential components of a rapid scanning FTIR spectrometer is given in Figure 1.2.1.

The light from an IR source is polychromatic and is sent to the beam splitter of a Michelson interferometer, which splits the beam of radiation into two and then recombines the two beams after introducing a path difference. This combined beam goes through the sample to the detector. The division of the beam is achieved with a beam splitter which transmits about 50% and reflects about 50%. One of the beams goes to a fixed mirror and the other to a mirror which can be moved to introduce a varying path difference. When the beams are recombined an interference pattern is obtained as the path difference is varied.

For a monochromatic source the interference pattern is a sine wave. This can be explained by relating the position of the movable mirror to the signal detected. When the movable mirror is in position 0, the path length of the reflected and transmitted light is the same. This means that the two parts of the recombined beam will be totally in phase and the detected signal will be a maximum (constructive interference, Figure 1.2.2). Motion of the movable mirror in either direction will change the path length of the transmitted beam. If the mirror is moved to position B or C (see Figure 1.2.1) the path difference between the two beams when they recombine would
be half a wavelength, one-quarter wavelength for each direction. Under this circumstance destructive interference will reduce the radiant power of the recombined beam to zero (Figure 1.2.3). Further motion to A and D will bring the two halves back in phase so that constructive interference can again occur. If the signal from the detector is plotted against the difference in path length of the two beams an interference pattern is produced which is sinusoidal. This is called an interferogram. The spacing between the maximum corresponds to a change in path difference equal to the wavelength. For a polychromatic source the interferogram is the resultant of the sinusoidal signals of the individual wave lengths emitted by the source (Figure 1.2.4). Only when the fixed and moving mirrors are equidistant are the sinusoidal signals due to each wavelength emitted by the source in phase, at this point the interferogram has a very large amplitude.

The normal frequency spectrum is obtained from the interferogram by Fourier transformation which is carried out by a microcomputer. Transmittance spectra are usually measured by first measuring a single-beam background spectrum and then a sample is placed in the beam and its single-beam spectrum is also measured. The ratio of these two spectra gives the transmittance of the sample. The negative logarithm of this spectrum is usually computed to yield the absorbance spectrum.

There are several advantages to measuring IR spectra interferometrically. Firstly the Multiplex (or Fellget) advantage which has already been mentioned and secondly the optical throughput or Jacquinot advantage; for spectra measured at the same resolution the energy throughput in an interferometer can be higher than in a dispersive spectrometer where it is restricted by the slits. In combination with the Fellget, advantage this leads to one of the most important features of an FTIR spectrometer, the ability to achieve the same SNR as a dispersive instrument in a much shorter time.

For an interferogram to be transformed into a spectrum it must be digitized. In essentially all modern FTIR spectrometers, this goal is achieved by sampling at equal intervals of optical path difference using the sinusoidal interferogram from a helium-neon laser beam which is passed through a different region of the beam-splitter of the interferometer. The IR interferogram is generally digitized once per wavelength of the laser interferogram (typically at a zero-crossing). This method of sampling gives rise to a third advantage of FTIR spectrometry; namely its extremely high wavenumber precision. A fourth benefit, which results in part from the good SNR and wavenumber precision of FTIR spectra, is the ease with which spectra can be manipulated in a computer.
CHAPTER TWO

Liquid and Solution Phase Spectral Study of Fatty Acid Methyl Esters

2.1 INTRODUCTION

The identification and quantitative analysis of the fatty acids, and more importantly of cis/trans unsaturated fatty acids, in fats and oils by IR spectroscopy is the main objective of this work. However, before IR spectroscopy could be applied to this specific field preliminary investigations were undertaken to establish essential correlations. This involved an in depth literature review and a comparative FTIR study of the IR spectra of FAME's.

A considerable number of papers have been published on the IR spectra of either specific bands corresponding to particular types of fatty acids or more general bands associated with particular fats and oils. The majority of these papers were published between 1940 and 1960 on dispersive instruments of poor resolution. Recently FTIR spectroscopy has begun to be extensively used in the analytical laboratory. This new interest can be attributed, in part, to the increased availability of complete commercial instruments. In addition, the advent of software which allows mathematical manipulation of the spectral data is also important. As a result of this data processing capability, advantage can be taken of the improved signal to noise ratio (SNR) available from FTIR yielding greater spectral sensitivity, see section 1.2. Thus the application of IR spectroscopy to qualitative/quantitative problems are substantially enhanced.

In this chapter the spectra of various FAME's (see Table 2.1.1) were recorded between 4000 and 400 cm⁻¹, in the liquid and solution phase using the FTIR spectrometer. A comparative study of the IR spectra obtained of the saturated and unsaturated FAME's was undertaken, with the objective of establishing whether FTIR could enhance spectral data differences, not observed using dispersive instruments, between the various types of FAME's; i.e. chain length, number of double bonds and configuration of double bonds.
Table 2.1.1

List of unsaturated FAME liquid phase spectra studied.
(Spectra can be found in volume II of this thesis, where the code applies)

<table>
<thead>
<tr>
<th>Spectrum code</th>
<th>FAME code</th>
<th>Spectrum code</th>
<th>FAME code</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP001</td>
<td>11:1Δ10</td>
<td>SP017</td>
<td>18:2Δ9t12t</td>
</tr>
<tr>
<td>SP002</td>
<td>12:1Δ11</td>
<td>SP018</td>
<td>18:3Δ6c9c12c</td>
</tr>
<tr>
<td>SP003</td>
<td>13:1Δ12</td>
<td>SP019</td>
<td>18:3Δ9c12c15c</td>
</tr>
<tr>
<td>SP004</td>
<td>14:1Δ9c</td>
<td>SP020</td>
<td>19:1Δ10c</td>
</tr>
<tr>
<td>SP005</td>
<td>14:1Δ9t</td>
<td>SP021</td>
<td>20:1Δ8c</td>
</tr>
<tr>
<td>SP006</td>
<td>16:1Δ9c</td>
<td>SP022</td>
<td>20:1Δ11c</td>
</tr>
<tr>
<td>SP007</td>
<td>16:1Δ9t</td>
<td>SP023</td>
<td>20:1Δ11t</td>
</tr>
<tr>
<td>SP008</td>
<td>18:1Δ6c</td>
<td>SP024</td>
<td>20:2Δ11c14c</td>
</tr>
<tr>
<td>SP009</td>
<td>18:1Δ6t</td>
<td>SP025</td>
<td>20:2Δ11t14t</td>
</tr>
<tr>
<td>SP010</td>
<td>18:1Δ9c</td>
<td>SP026</td>
<td>20:3Δ8c11c14c</td>
</tr>
<tr>
<td>SP011</td>
<td>18:1Δ9t</td>
<td>SP027</td>
<td>20:3Δ11c14c17c</td>
</tr>
<tr>
<td>SP012</td>
<td>18:1Δ11c</td>
<td>SP028</td>
<td>22:1Δ13c</td>
</tr>
<tr>
<td>SP013</td>
<td>18:1Δ11t</td>
<td>SP029</td>
<td>22:1Δ13t</td>
</tr>
<tr>
<td>SP014</td>
<td>18:1Δ9c12(Oh)</td>
<td>SP030</td>
<td>22:4Δ7c10c13c16c</td>
</tr>
<tr>
<td>SP015</td>
<td>18:1Δ9t12(Oh)</td>
<td>SP031</td>
<td>24:1Δ15c</td>
</tr>
<tr>
<td>SP016</td>
<td>18:2Δ9c12c</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
2.2 REVIEW ON INFRARED SPECTROSCOPY OF LIPID PRODUCTS

This review is intended to give essential background into the advances made in the interpretation of spectral bands which are of significance to the lipid chemist. It is not intended to include the definition or principles of IR spectroscopy as this subject has been adequately covered in standard texts and in general reviews (Straughan et al., 1976). The emphasis of this review will be directed at methyl ester derivatives of fatty acids, because of the reasons given in section 1.1.8 and due to the lower boiling point of the methyl esters over the fatty acids, which has resulted in their exclusive use in GC analysis. The IR spectroscopy of fatty acids and triglycerides will also be covered briefly.

The first recorded spectra of fatty acids and vegetable oils were published in 1905 by Coblentz in the first so-called "library" of IR spectra. Fifteen years later, a technical article devoted exclusively to the application of IR spectroscopy to fatty acid products was published by Gibson (1920). However, it was not until the decade 1940-1950 that IR spectroscopy was introduced to the lipid chemist as an analytical tool, resulting in the publication of 23 articles on the application of IR to this field. Since 1950 there has been a widespread acceptance of the technique by the lipid chemist and accordingly an increase in the number of papers describing IR spectral procedures for qualitative identification, quantitative determination, and for the elucidation of structures of lipid material.

In all about 100 IR absorption bands are employed in the study of fats and oils (O'Connor, 1956) and several review articles dealing with IR spectroscopy for their general analysis have been published (Wheeler, 1954; Chapman, 1965; Freeman, 1968; Kochhar, 1987). In general lipid chemists have interpreted IR spectra in terms of characteristic group frequencies. Attention was focused on bands associated with specific molecular structures, and the rest of the spectrum disregarded except for empirical fingerprint identification. Classification of vibrational modes via normal coordinate analysis has been applied only to small symmetrical molecules, and to polymers with high symmetry in the repeat unit. Most organic molecules are too large and asymmetrical for such qualitative attempts at a detailed analysis of their vibrational spectra. This is true of the long chain fatty acids which have been studied. Assignments of the vibrational modes of formic acid, acetic acid and various simple unsaturated hydrocarbons calculated using this technique have been reviewed (Sverdlov, 1974).

An alternative technique used to interpret the vibrational spectra of more complex molecules involves the transposition of the results of normal coordinate analysis of simpler molecules, aided
by qualitative comparison of the spectra of isotopically substituted species. This method of assignment has become widely accepted in publications on the IR of large molecules. A particularly illuminating study by Furrer et. al. (1972) on "normal modes and group frequencies-conflict or compromise?"; casts a critical eye on both techniques described above.

Selective deuteration shifts the wavenumber of the vibration of the substituted groups to lower values, according to the approximate isotopic frequency rule (Krimm, 1960a). This has been used, as mention above, to ascertain vibrational modes and also to indicate their coupling. This method has frequently been used for smaller molecules. Among the fatty acids, deuteriated species were investigated both for formic acid (Millikan, 1957 & 1958; Sverdlov, 1953a & 1953b) and acetic acid (Haurie, 1965a & 1965b) in the monomer, dimer and crystalline forms. Among esters, methyl and ethyl formate/acetate and their deuteriated species were investigated in solution (Nolin, 1956a & 1956b). Further studies in solution were conducted on methyl dodecanoate (laurate) and its analogues deuterated in the α and ω positions and in the ester group (Jones, 1962). From an analysis of these spectra the assignment of a considerable number of the bands were made to localized group vibrations and verification of the existence of so-called "zone frequencies" was made, where two of the groups are found to be coupled. Methyl stearate specifically deuteriated around the middle of the chain was investigated in solution to provide a basis for quantitative determinations of CHD and CD₂ contents in unknown samples (Rohwedder, 1967).

The solid state spectra of saturated long chain fatty acids and esters have strong evenly spaced peaks between the bands of constant positions at 1380 cm⁻¹ and 1170 cm⁻¹. These bands are often referred to as the "progression bands" and from the number of bands occurring the chain length can be inferred. Jones, McKay and Sinclair (1952) were the first to draw attention to this spectral feature. Brown et. al. (1954a and 1954b) assigned these bands to wagging and/or twisting modes. Similar bands were assigned in the spectra of hydrocarbons, however, the bands were of weaker intensity (Schachtschneider, 1963; Synder, 1963). Confirmation of the wagging and twisting modes and group frequencies as well as various assignments of bands were made by Dinh Nguyen and Fischmeister (1970a and 1970b) in their comparative IR study of methyl stearate and various specifically deuteriated analogues in the solid state. Their studies revealed that deuteration of both the α and β-methylene group interrupts the coupling between the methylene chain modes and C-O stretching modes, resulting in the loss of either intensity or bands in the progression region. This explains why the solid state IR spectra of hydrocarbons which contain no polar groups had progression bands of low intensity. The introduction of the
substituent in different positions along the chain length of methyl stearate was found to disrupt
the coupling of the chain absorption so that only the segment carrying the polar group retains its
strong absorption, while the other one absorbs weakly. It was found that the progression bands
observed, which were due to the chain segment with the polar group, resembled that of the
corresponding short chain ester.

Although the solid state IR spectra of fatty acids and esters provide more spectral detail than the
corresponding liquid or solution phase spectra, this type of investigation has not really taken off.
This is partially due to the fact that the spectra obtained are dependent upon the crystal form,
which is complicated by the occurrence of polymorphism (see section 1.1.4). Also there are
problems of band overlap encountered in mixtures containing a wide spread of chain lengths.
Meiklejohn et al. (1957) examined the spectra of soaps as well as free fatty acids by a potassium
bromide disk technique. This avoided melting the crystallized fatty acids and produced
reproducible spectra from the same batch, but did not overcome the problem of different crystal
structures in different batches. Attempts at determining the chain length of mixtures were limited
to two fatty acids in the ratio 1:1. This was achieved by visually unscrambling the two sets of
uniformly spaced bands.

This chapter is predominantly concerned with the liquid phase spectra of FAME's which are similar
to those in solution. In both cases the free rotation of the methylene chain causes different
conformations to absorb simultaneously, giving a broad smoothed-out chain absorption observed
mainly in the wagging modes, with only small variations occurring in the relative band intensities
(Sinclair et al., 1952a). Despite the loss in IR information, more papers have been published on
the spectra of fatty acids or ester in solution or the liquid phase than in the solid. This is due to the
reasons outlined in the previous paragraph and because quantitative work is difficult in the solid
state. A more specific description of the spectral features common to saturated, cis-, trans- and
conjugated FAME's in the liquid or solution phase follows.

2.2.1 Saturated FAME's

In order to simplify the task of assigning the IR bands that occur in saturated FAME's, the spectrum
of methyl stearate has been selected to represent this category. The spectrum of methyl stearate
is conveniently divided into the following two regions:

Region 1; 4000 cm\(^{-1}\) to 1600 cm\(^{-1}\)
Region 2; 1600 cm\(^{-1}\) to 400 cm\(^{-1}\)

Each of these regions is represented by a spectrum of the region (Figures 2.2.1.1 and 2.2.1.2), indicating the locations of each band, and a table (Tables 2.2.1.1 and 2.2.1.2) showing the frequency, assignment and reference source of each band. Greek letters identify bands that are not observed in the methyl stearate spectrum but are presumed to be present from the analysis of the spectra of the deuterated derivatives.

**Table 2.2.1.1**

Frequency, assignment and reference source of each band in the spectral region 4000 cm\(^{-1}\) to 1600 cm\(^{-1}\) of methyl stearate (Figure 2.2.1.1), representative of saturated FAME's. See Table 2.2.1.2 for details of the references.

<table>
<thead>
<tr>
<th>Band</th>
<th>(\nu_{\text{max}}) (cm(^{-1}))</th>
<th>Group</th>
<th>Assignment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>3028</td>
<td>-COOCH(_3)</td>
<td>asym. C-H stretch ((a'))</td>
<td>1, 2</td>
</tr>
<tr>
<td>B</td>
<td>2995</td>
<td>-COOCH(_3)</td>
<td>asym. C-H stretch ((a''))</td>
<td>1, 2</td>
</tr>
<tr>
<td>(\beta)</td>
<td>2955</td>
<td>-COOCH(_3)</td>
<td>sym. C-H stretch</td>
<td>1, 2</td>
</tr>
<tr>
<td>C</td>
<td>2950</td>
<td>-CH(_3)</td>
<td>asym. C-H stretch</td>
<td>1, 13, 14</td>
</tr>
<tr>
<td>D</td>
<td>2922</td>
<td>-(CH(<em>2))(</em>{15})</td>
<td>asym. C-H stretch</td>
<td>1, 13, 14</td>
</tr>
<tr>
<td>E</td>
<td>2870</td>
<td>-CH(_3)</td>
<td>sym. C-H stretch</td>
<td>1, 13, 14</td>
</tr>
<tr>
<td>F</td>
<td>2852</td>
<td>-(CH(<em>2))(</em>{15})</td>
<td>sym. C-H stretch</td>
<td>1, 13, 14</td>
</tr>
<tr>
<td>G</td>
<td>1742</td>
<td>-COOCH(_3)</td>
<td>C=O stretch</td>
<td>1</td>
</tr>
</tbody>
</table>
Table 2.2.1.2

Frequency, assignment and reference source of each band in the spectral region 1600 cm\(^{-1}\) to 400 cm\(^{-1}\) of methyl stearate (Figure 2.2.1.2), representative of saturated FAME's.

<table>
<thead>
<tr>
<th>Band</th>
<th>(v_{\text{max}}) (cm(^{-1}))</th>
<th>Assignment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Group</td>
<td>Mode</td>
</tr>
<tr>
<td>H</td>
<td>1467</td>
<td>(-(\text{CH}<em>2)</em>{15})-</td>
<td>band a; C-H scissor</td>
</tr>
<tr>
<td></td>
<td></td>
<td>-CH(_3)</td>
<td>band b; asym. C-H bend</td>
</tr>
<tr>
<td></td>
<td>1458</td>
<td>-CH(_3)</td>
<td>band k; C-H bend ((a^*))</td>
</tr>
<tr>
<td>n</td>
<td>1440</td>
<td>(-(\text{CH}<em>2)</em>{15})-</td>
<td>C-H scissor or wag</td>
</tr>
<tr>
<td>J</td>
<td>1436</td>
<td>-COOCH(_3)</td>
<td>asym. C-H bend</td>
</tr>
<tr>
<td>K</td>
<td>1419</td>
<td>-CH(_2)-</td>
<td>C-H scissor</td>
</tr>
<tr>
<td>L</td>
<td>1378</td>
<td>-CH(_3)</td>
<td>sym. C-H bend</td>
</tr>
<tr>
<td>z</td>
<td>1368</td>
<td>\ -(\text{CH}<em>2)</em>{15})-</td>
<td>C-H wag or twist</td>
</tr>
<tr>
<td>M</td>
<td>1362</td>
<td>(\alpha)-CH(_2)-</td>
<td>C-H wag</td>
</tr>
<tr>
<td>x</td>
<td>1352</td>
<td>\ -(\text{CH}<em>2)</em>{15})-</td>
<td>C-H wag or twist</td>
</tr>
<tr>
<td>N</td>
<td>1340</td>
<td>\ -(\text{CH}<em>2)</em>{15})-</td>
<td>C-H wag or twist</td>
</tr>
<tr>
<td>O</td>
<td>1305</td>
<td>\ -(\text{CH}<em>2)</em>{15})-</td>
<td>C-H twist (or wag?)</td>
</tr>
<tr>
<td>P</td>
<td>1245</td>
<td>-CH(_2)COOCH(_3)</td>
<td>C-O skeletal coupled with (\alpha)-CH(_2)- deformation</td>
</tr>
<tr>
<td>Q</td>
<td>1196</td>
<td>-CH(_2)COOCH(_3)</td>
<td>C-O skeletal coupled with (\alpha)-CH(_2)- deformation</td>
</tr>
<tr>
<td>R</td>
<td>1169</td>
<td>-CH(_2)COOCH(_3)</td>
<td>C-O skeletal coupled with (\alpha)-CH(_2)- deformation</td>
</tr>
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<td>1112</td>
<td>\ -(\text{CH}<em>2)</em>{15})-</td>
<td>C-C skeletal coupled with end groups</td>
</tr>
<tr>
<td>T</td>
<td>1074</td>
<td>\ -(\text{CH}<em>2)</em>{15})-</td>
<td>C-C skeletal</td>
</tr>
<tr>
<td>U</td>
<td>1016</td>
<td>-CH(_2)COOCH(_3)</td>
<td>C-O skeletal coupled</td>
</tr>
<tr>
<td>V</td>
<td>875</td>
<td>-COOCH(_3)</td>
<td>methyl rock?</td>
</tr>
<tr>
<td>W</td>
<td>845</td>
<td>-COOCH(_3)</td>
<td>methyl rock?</td>
</tr>
<tr>
<td>X</td>
<td>755</td>
<td>\ -(\text{CH}<em>2)</em>{15})-</td>
<td>C-H rock</td>
</tr>
<tr>
<td>Y</td>
<td>721</td>
<td>\ -(\text{CH}<em>2)</em>{15})-</td>
<td>C-H rock</td>
</tr>
</tbody>
</table>
Information derived from the analysis of FAME's of different chain lengths was also used to assign these bands. Studies by Jones (1952a) on methyl laurate and some of its deuteriated derivatives, were of particular use in the assignment of bands. Another paper by Jones (1952b) on the solution spectra of various different chain length FAME's, including methyl stearate, investigated the effects of the chain length on the peak intensity of the bands assigned in Table 2.2.1.2. These studies provide further evidence toward the assignments corresponding to methyl stearate in the above tables. The latter reference also discusses the "zone concept", which assumes that each of the twenty five bands assigned in the tables can be attributed to a localised group vibration which is either strictly localised within a particular zone (21 bands as indicated on figure 2.2.1.3), or is due to coupling between vibrations in different zones (4 bands). For example, bands associated with zones A, C and D, which are localised, should be unaffected in position and intensity with increase in chain length. On the other hand, zone B bands involve purely internal vibrations of each methylene group which should increase in intensity without wavenumber shift when the chain length is increased. In those modes that involve coupling between successive methylene groups, progressive wavenumber shifts may occur.

The influence of chain length upon the bands in the region 4000 cm⁻¹ to 2000 cm⁻¹ of triglycerides, mono-glycerides and methyl esters in solution has been studied by Keeney (1962). A linear correlation was observed between the relative absorption intensity at 2922 cm⁻¹ (methylene C-H stretch) to 1742 cm⁻¹ (ester C=O stretch) versus the carbon chain length of the FAME's. This has been used to establish the average length of the carbon chain of saturated FAME mixtures. Values obtained by this means were found to be in good agreement with values obtained by GC. However, this method is limited to saturated systems because double bonds have a marked effect upon the methylene stretch absorption.
2.2.2 Cis FAME's

The absorption bands associated with the methylene groups of the chain, the terminal methyl group, the carboxy and carbomethoxy groups observed in cis-FAME's are similar to those discussed in the previous section for the saturated FAME's. This section will be concerned only with those features of the IR spectra which are associated with the cis-ethylenic group. Absorption specific to this group in FAME's occur in the following three regions of the spectrum - 3000 to 3100 cm⁻¹, 1580 to 1680 cm⁻¹, and 690 to 980 cm⁻¹ - corresponding to the stretching vibration C-H of the C=C-H group, stretching vibration of C=C bond and out of plane bending vibration of the C=C-H carbon hydrogen bonds respectively. The assignment of these bands will be discussed in the following pages. In addition, bands which either appear in the spectra of cis-FAME's but have conflicting assignments, or bands which have only been observed in specific cis-FAME's will be discussed separately.

The 3000 to 3100 cm⁻¹ region; Two assignments have been reported for the absorption band at 3020 cm⁻¹ associated with a double bond mode. Sinclair et al. (1952b) assigned this band to the =C-H stretching vibration of an ethylenic double bond in their study of the IR spectra of various unsaturated fatty acids and their methyl esters. They also showed that the intensity of this band increased with the number of cis double bonds present, while the relative intensity of the methylene C-H stretching vibration diminished. These observations provided a basis for the evaluation of the degree of cis unsaturation.

The assignment of the band at 3020 cm⁻¹ to the C-H stretching vibration of alpha methylene groups adjacent to the double bond was made by Adams and Auxier (1951). This conclusion was reached on the basis of their observations that the intensity of this band decreases simultaneously with the oxidation of dipentaerythritol linoleate, and that the intensity of this band was very small in oleate, greater in linoleate, and still greater in linolenate. Further support for this explanation was provided by Privett et al. (1953) based on their observations on autoxidized methyl linoleate in which the band at 3020 cm⁻¹ was much weaker in the conjugated peroxide

\[-CH_2-CH=CH-CH=CH-\]

\[\text{OOH}\]

product (having the assumed structure shown above, which has one alpha methylene compared to two for oleate or conjugated linoleate and three for linoleate) than in oleate or cis-trans...
conjugated linoleate. This is in accordance with assignment of this band to the alpha methylene group.

Finally, the most conclusive evidence towards the assignment of this band was provided by Hirabayashi et al. (1971). They studied the IR spectra of various unsaturated methyl esters including those of 9,10-dideutero-oleate and 9,10-dideutero-elaidate in solution. Quantitative studies in solution showed that the ratio between the intensities of the band at 3020 cm\(^{-1}\) in various unsaturated FAME's relative to that of methyl oleate, are almost equal to the ratio of the numbers of the cis-ethylenic double bonds present in the molecule. There was no apparent difference between isolated and conjugated systems. Trans FAME's studied were found not to possess prominent absorption bands at 3020 cm\(^{-1}\) though they, like the cis FAME's, have alpha methylene groups adjacent to the double bond. In addition, the deuterated compounds (-CD=CD-) which all have alpha methylene groups, did not possess an absorption band at 3020 cm\(^{-1}\). Hirabayashi et al. therefore concluded that the band at 3020 cm\(^{-1}\) in the spectra of methyl esters can be attributed to the =C-H stretching vibration of cis-ethylenic double bonds.

1580 to 1680 cm\(^{-1}\) region; IR spectral studies on a number of long chain saturated and unsaturated fatty acids, triglycerides, alcohols and in particular methyl esters have revealed that in all of these four classes the cis-unsaturated compounds have a slight broadening or inflexion on the long wavelength side of the strong carbonyl band at approximately 1660 cm\(^{-1}\). This band was assigned to the cis C=C stretching vibration as no band or inflexion was observed for the trans C=C stretching mode (Shreves et al., 1950; Sinclair et al., 1952b). In the spectra of methyl esters the C=C stretching band also occurs at 1660 cm\(^{-1}\), but it is better resolved as there is less overlap with the ester carbonyl band at 1741 cm\(^{-1}\).

The above observations arise from the fact that IR absorption takes place only when there is a change of dipole moments. There is no appreciable change involved during the C=C stretching vibration of a trans symmetrical molecule. On the other hand, some change in the dipole moment occurs in compounds having cis double bonds, whilst both types are Raman active (Sheppard and Simpson, 1952; Sheppard, 1951).

McCutcheon et al. (1941) used the above theoretical concept to show that oleic acid has a cis-configuration, linoleic a cis-cis, and linolenic a cis-cis-cis structure. Elaidic acid similarly was shown to be a trans isomer and linelaaidic to have a trans-trans configuration. Evidence for these structures was provided by the corresponding IR spectra of the ethyl esters in the region 2000 to 1430 cm\(^{-1}\) which either showed the presence or absence of the cis C=C stretching band. Raman
data confirm these results and suggest that the trans C=C stretch gives rise to a band at a slightly higher frequency ($\approx 1670 \text{ cm}^{-1}$, Sheppard, 1952; McCutcheon, 1941).

No intensity studies have been conducted on the C=C stretching vibration of unsaturated long chain methyl ester or related compounds in the IR. This has been due to the difficulties associated with the measurement of a band of low intensity which overlaps with a strong band. However, according to theory, the intensity of the C=C double bond in the IR would be expected to diminish when it is moved from the end of a chain toward the centre because the molecule would become more symmetrical. Wotiz and Miller (1949) have observed this effect in the case of the C=C stretching vibration. Further evidence to support this supposition was provided by Shreve et al. (1950), who observed that 10-undecenoic acid and methyl ester both showed relatively strong C=C absorptions in the IR due to the terminal vinyl grouping. Bellamy (1988) has presented an excellent review on the C=C stretching vibration of various compounds, concentrating on factors such as symmetry considerations, conjugation and substitution, all of which influence both the frequency and intensity of this band.

690 to 980 cm$^{-1}$ region; The association of a band near 690 cm$^{-1}$ with the analogous vibration of the cis double bond, which overlaps with the methylene rocking band at 720 cm$^{-1}$, has been suggested in various papers (Shreves et al., 1950; Jones, 1950; Sinclair et al., 1952b; Aslers et al., 1953; Barnard et al., 1950; Thompson et al., 1948). Sheppard and Simpson (1952) tentatively assigned this band to the hydrogen out-of-plane deformation about the double bond. The position of this band is more variable than that of the corresponding trans band.

Other regions; This section will cover bands which have been observed in the spectra of cis FAME's but have been overlooked because of their weak intensity or overlap with other bands, thus, making them difficult to use for diagnostic purposes or quantitative work.

The first of these bands was reported by Sinclair et al. (1952b) who observed a band at 1435 cm$^{-1}$ in the solid phase spectra of unsaturated fatty acids (including elaic acid) the intensity of which increased progressively with the degree of unsaturation. This band was tentatively assigned to a bending vibration of a methylene group adjacent to a double bond. Other studies on cis and trans disubstituted ethylenes, including deuteration studies, have shown that in-plane $\equiv$C-H deformation in alkenes usually absorbs in the range 1420-1385 cm$^{-1}$ for cis olefins and 1310-1280 cm$^{-1}$ for trans olefins. This difference has been confirmed by Raman data where the in-plane deformation can be identified with more certainty due to its considerable intensity in
both isomers (Hoffmann, 1958; Sheppard and Simpson, 1950). Examination by the present
author of spectra of methyl esters published by Sinclair et al. (1952b) showed that the cis
unsaturated esters all possess a band at ~1400 cm$^{-1}$, a band not observed in the spectrum of
methyl elaidate. Further studies are therefore required to confirm the assignment of this band in
the cis and trans isomers.

Finally Jackson et al. (1952) reported a band in their IR spectral studies of non-conjugated and
conjugated isomers of methyl linoleate in the region of olefinic C-H bending. They observed a
weak band with a maximum at 913 cm$^{-1}$ which was found only in the spectrum of methyl linoleate
(18:2Δ9c12c). No attempt was made to assign the band.

2.2.3 Trans FAME's

As in the case of cis FAME's certain features of the spectra of trans FAME's are similar to those of
the saturated FAME's (see section 2.2.1). The spectral features associated with the trans
ethylene group will be discussed in this section. These occur in the regions - 3000 to 3100 cm$^{-1}$,
1580 to 1680 cm$^{-1}$, and 690 to 980 cm$^{-1}$.

3000 to 3100 cm$^{-1}$ region; The cis FAME's possess a band in this region at 3020 cm$^{-1}$
 assigned to the =C-H stretch of the cis ethylenic double bond. The corresponding band has not
been reported in the literature for the trans FAME's. However, a band at about 3030 cm$^{-1}$ was
observed in the spectrum of methyl linoleate (18:2Δ9t12t) by Hirabayashi et al. (1971), the
intensity of which was considerably lower than that of the corresponding cis isomer.

1580 to 1680 cm$^{-1}$ region; Shreve et al. (1950) were unable to detect the C=C stretching
vibration in trans long-chain unsaturated fatty acids, methyl esters and alcohols. The
 corresponding cis isomers possess a band at 1654 cm$^{-1}$. The absence of the band in the trans
isomers is due to the symmetry considerations described in the previous section.

690 to 980 cm$^{-1}$ region; The association of a prominent band at 965 to 975 cm$^{-1}$ with the
trans double bond is well established (Barnard et al., 1950; Rausmussen et al., 1947; Kilpatrick
and Pitzer, 1947) and has been used to differentiate between cis and trans-unsaturated fatty
acids and esters (Lemon and Cross, 1949; Rao and Daubert, 1948; Shreves et al., 1950). This
band has been assigned to the –C–H deformation about a trans ethylenic double bond (Sheppard and Sutherland, 1949). In a review on the "analysis of oils and fats using infrared spectroscopy" by Kochhar and Rossell (1987), the authors claim that the band arises from the deformation of the C–H bonds adjacent to an isolated trans double bond. Presumably this refers to the C–H bonds marked * in the figure below, though this is not made clear from the author's statement. No reference is given to substantiate this assignment.

\[ \text{R}_1\text{C}^*\text{H}_2\text{CH} = \text{CHC}^*\text{H}_2\text{R}_2 \]

Measurement of this absorption band under controlled conditions is the basis for the various quantitative methods used to establish trans content. These methods will be discussed in Chapter 3.

Other trans bands; In the previous section (2.2.2) a band observed in the spectra of cis compounds occurring at 1400 cm\(^{-1}\) was tentatively attributed to the in-plane hydrogen deformation about a cis double bond. The corresponding band in the trans fatty acid compounds has not been reported in the literature. This is principally due to the fact that symmetry considerations either forbid the appearance of the vibration in certain cases (i.e. when symmetrically substituted) or result in a weakening of the intensity in the IR.

Sheppard and Sutherland (1949) have shown that a strong band appears in the region 1310-1290 cm\(^{-1}\) in both the Raman and IR spectra of a series of trans alkyl substituted ethylenes. They have assigned these to the two in-plane deformations \(v_1\) and \(v_2\) with different symmetry classes. Other authors have reported a similar band in the IR spectrum of unsaturated hydrocarbons of this type (Sheppard and Simpson, 1952; Sheppard, 1951), but the intensity appears to be somewhat variable and the band is occasionally weak in intensity. It has not been detected in the spectra of long chain trans unsaturated fatty acid compounds.

2.2.4 Conjugated unsaturated FAME's

Spectral studies on the conjugated compounds have predominantly concentrated on the region 1000 to 900 cm\(^{-1}\), where the distinctive band patterns characteristic of various arrangements of cis and trans groups occur. The information available about the band patterns of conjugated
ethylenic bonds is summarised in Table 2.2.4.1.

Examination of this table reveals that there is a progressive shift from 965 cm\(^{-1}\) (isolated trans group) to 997 cm\(^{-1}\) (four conjugated trans groups). This hypochromic shift has been noted by various authors (O'Connor, 1956; Colthup, 1971; Ahlers et al., 1953), who have predicted that higher orders of trans conjugated polyenes will have an absorption band with a limit near 1000 cm\(^{-1}\). It is also apparent from the table that if one or two cis groups are conjugated with the trans groups, the comparable polyene band is also shifted to a higher wavenumber, but to a lesser extent. Furthermore, a second band is normally observed at a lower wavenumber when a cis group is present. Colthup (1971) has presented an interpretation of the bands in this region and also predicted absorption patterns, including intensities, for less commonly observed polyenes with cis and trans conjugated groups.

Reports of bands assigned to the conjugated cis and trans compounds in other spectral regions include, studies of the band at \( \approx 3030 \) cm\(^{-1}\), attributed to the \(-C-H\) stretch, of cis-trans isomers of the methyl linoleate (Privett et al., 1953; Hirabayashi et al., 1971), and the very weak band at \( \approx 1600 \) cm\(^{-1}\) observed by Sparreboom (1956) in the spectra of the cis-cis conjugated isomers of methyl linoleate (18:2\(\Delta 9\)c11c & 18:2\(\Delta 10\)c12c). This absorption was attributed to the C=C stretching mode, in which the conjugated double bonds vibrate out-of-phase with respect to one another.

An elegant spectral study of the various isomers of sorbic acid (hex-2,4-dienolc) by Allan et al. (1955), in which the intensity of the C=C stretching vibration is increased due to conjugation with the carbonyl group, revealed two bands ascribed to the C=C stretching vibrations of the cis-cis isomer. Two bands attributed to the C=C stretch were also observed in the spectrum of cis-cis isomer of methyl deca-2,4-dienoate by Crombie (1955). In both the cis-cis isomers mentioned above, the intensities of the C=C stretching bands are enhanced due to conjugation with the carbonyl group and the higher frequency band was of greater intensity than that of the lower frequency band. In the cis-cis conjugated isomers of methyl linoleate studied by Sparreboom (1953) the double bonds are located near the centre of the methylene chain, which decreases the intensity of the C=C stretching modes due to symmetry considerations. This may explain why the second C=C stretching band, present in the spectra of conjugated double bonds adjacent to a carbonyl, was not observed by Sparreboom. Table 2.2.4.2 shows the positions of the C=C stretching bands assigned to various conjugated isomers.
### Table 2.2.4.1

Out-of-plane bending frequencies of conjugated cis and trans double bonds (\(-\text{C-H bands}\))

<table>
<thead>
<tr>
<th>Isomers</th>
<th>Position of band(s) cm(^{-1})</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>-CH(<em>{\text{i}})-CH(</em>{\text{j}})- isolated</td>
<td>965 s</td>
<td>1</td>
</tr>
<tr>
<td>-CH(<em>{\text{i}})=CH(</em>{\text{j}})- isolated</td>
<td>ca. 700 m</td>
<td>1</td>
</tr>
<tr>
<td>-CH(<em>{\text{i}})=CH(</em>{\text{j}})-CH(_{\text{k}})-</td>
<td>988 s</td>
<td>2, 3, 4, 5, 6, 7, 8</td>
</tr>
<tr>
<td>-CH(<em>{\text{i}})=CH(</em>{\text{j}})-CH(<em>{\text{k}})=CH(</em>{\text{j}})-</td>
<td>982 s, 948 m</td>
<td>2, 4, 5, 6, 7, 8</td>
</tr>
<tr>
<td>-CH(<em>{\text{i}})=CH(</em>{\text{j}})-CH(<em>{\text{k}})=CH(</em>{\text{j}})+</td>
<td>994 s</td>
<td>2, 4, 8</td>
</tr>
<tr>
<td>-CH(<em>{\text{i}})=CH(</em>{\text{j}})-CH(<em>{\text{k}})=CH(</em>{\text{j}})-</td>
<td>991 s, 965 m, 930 w</td>
<td>8</td>
</tr>
<tr>
<td>-CH(<em>{\text{i}})=CH(</em>{\text{j}})-CH(<em>{\text{k}})=CH(</em>{\text{j}})-</td>
<td>986 m, 938 m</td>
<td>8</td>
</tr>
<tr>
<td>-CH(<em>{\text{i}})=CH(</em>{\text{j}})-CH(<em>{\text{k}})=CH(</em>{\text{j}})-</td>
<td>997 s</td>
<td>2, 8</td>
</tr>
<tr>
<td>-(CH(<em>{\text{i}})=CH(</em>{\text{j}}))(_{\text{n}}) ((n &gt; 4))</td>
<td>increases with (n), upper limit (= 1000)</td>
<td>2, 8, 9, 3</td>
</tr>
</tbody>
</table>

Table 2.2.4.2
The C=C stretching bands of various conjugated isomers

<table>
<thead>
<tr>
<th>Conjugated isomers</th>
<th>C=C stretching bands (cm⁻¹)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃-CH₃CH-CH-CH₂CO₂-CH₃</td>
<td>1642, 1614</td>
<td>Allan et al. (1955)</td>
</tr>
<tr>
<td>CH₃-CH₃CH₂CH₂CH₀₂-CH₃</td>
<td>1639, 1597</td>
<td>Allan et al. (1955)</td>
</tr>
<tr>
<td>CH₃-CH₂CH₂CH₀₂-CH₃</td>
<td>1634, 1598</td>
<td>Allan et al. (1955)</td>
</tr>
<tr>
<td>CH₃-CH₂CH₂CH₀₂-CH₃</td>
<td>1623, 1587</td>
<td>Allan et al. (1955)</td>
</tr>
<tr>
<td>CH₃-CH₀₂CH₀₂-CH₃</td>
<td>1659</td>
<td>Allan et al. (1955)</td>
</tr>
<tr>
<td>CH₃-CH₀₂CH₀₂-CH₃</td>
<td>1644</td>
<td>Allan et al. (1955)</td>
</tr>
<tr>
<td>CH₃-C≡C-CH₀₂-CH₃</td>
<td>1615</td>
<td>Allan et al. (1955)</td>
</tr>
<tr>
<td>CH₃-C≡C-CH₀₂-CH₃</td>
<td>1612</td>
<td>Allan et al. (1955)</td>
</tr>
<tr>
<td>CH₃-CH₀₂CH₀₂-CH₃</td>
<td>1626</td>
<td>Allan et al. (1955)</td>
</tr>
<tr>
<td>CH₃-CH₀₂CH-CH≡C-CH₀₂-CH₃</td>
<td>1615</td>
<td>Allan et al. (1955)</td>
</tr>
<tr>
<td>CH₃-(CH₂)₄-CH₀₂-CH₃</td>
<td>1647, 1618</td>
<td>Crombie (1955)</td>
</tr>
<tr>
<td>CH₃-(CH₂)₄-CH₀₂-CH₃</td>
<td>1639, 1607</td>
<td>Crombie (1955)</td>
</tr>
<tr>
<td>CH₃-(CH₂)₄-CH₀₂-CH₃</td>
<td>1640, 1602</td>
<td>Crombie (1955)</td>
</tr>
<tr>
<td>CH₃-(CH₂)₄-CH₀₂-CH₃</td>
<td>1634, 1592</td>
<td>Crombie (1955)</td>
</tr>
</tbody>
</table>
2.3 EXPERIMENTAL

2.3.1 Perkin Elmer 1750 FTIR system

Figure 2.3.1 is a schematic diagram of the Perkin Elmer 1750 infrared Fourier transform spectrometer used to obtain the data throughout the present work. The FTIR spectrometer comprises of the following:

Optical unit; encapsulates a single beam Michelson interferometer with rotary scan and bidirectional data collection which acquires a full double sided interferogram. The operation of the FTIR interferometer has been outlined in Section 1.2. The interferometer compartment is sealed and desiccated to give an ultra high background stability. Further background stability was provided by purging the path lengths of sample and detector areas with dry nitrogen gas (N₂ dried through molecular sieve) via a special port.

Microprocessor control and data processing system; interferograms produced by the optical unit are converted to spectra via Fourier transformation in this unit.

Visual display unit (VDU) and control keyboard; this unit permits operator control of the instrument and a limited number of data handling routines.

Model PP1 plotter printer; enables plots of the spectra to be obtained.

Perkin Elmer 7000 series professional computer and PECDS - 3 IR software; like the VDU and control keyboard mentioned above this unit provides instrumental control, however, the data handling facilities are more extensive. The most important of these data handling facilities is the PECDS-3 IR application software which is an advanced IR data processing package. PECDS-3 commands can be strung together in a sequence, called an Obey program, which can perform calculations, take spectra, store data, make judgements, spectral manipulation etc. Obey programs allow automation of an analysis and have been extensively used by the present author. Examples of Obey programs with explanations of their function can be found in Appendix II. A total of 102 PECDS-3 commands exist at present, the reader is referred to the Perkin Elmer 1700 series FTIR spectrometer CDS-3 operator manual for a full listing and description of their functions.
2.3.2 FTIR scanning parameters

The following parameters were used to obtain the FAME spectra in this chapter:

- Apodisation - Norton-Beer (medium setting)
- Jacquinot stop - 1
- Resolution - 2 cm\(^{-1}\)
- Spectral smoothing - none
- Gain - 1
- Scan speed - slow
- Magnitude spectrum - no
- Background phase - no
- Number of scans - 50
- Scanning range - 4000 to 400 cm\(^{-1}\)
- Detector - fast recovery deuterated triglycine sulphate detector with CsI windows
- Scanning mode - interleaved scanning which consists of a series of cycles of background and sample scans. In each cycle, the average of the sample spectra is ratioed against the average of the background spectra, compensating for any background changes.

Further details of the various parameters mentioned above can be found in the Perkin Elmer manual "1700 series IR Fourier transform spectrometers operator's manual". The IR spectra of the FAME's in the liquid phase were examined over the range 4000 to 400 cm\(^{-1}\) as very thin films between NaCl or KBr plates with or without a 25 \(\mu\)m teflon spacer. Studies on the bands of low intensity were conducted on the FAME spectra produced using the spacer, while bands of high intensity were studied using the spectra produced with no spacer.

Some of the longer chain length saturated and trans-unsaturated FAME's were solids at room temperature, and were sampled in the liquid phase by using pre-heated cells (10 minutes in an oven at 40\(^{\circ}\)C) which melted the samples. The number of scans taken was reduced to 10 to
ensure solidification did not take place during the sampling. The procedures used to obtain spectra of FAME's in solution (CCl₄) are outlined in Chapter 3.

2.3.3 Source of purchased FAME's

The purity of the FAME's studied were generally > 99% and purchased from two sources; Sigma Chemicals Co. Ltd., Fancy Rd., Poole, Dorset, BH17 7TG, UK. and Bast of Copenhagen, Biological Division, 44 Ingerlarsgrade, DK-1705, Copenhagen V, Denmark. The long chain unsaturated alcohols studied were purchased from Sigma. In cases where decomposition was suspected the FAME in question was analysed by the capillary GC method described in Section 2.3.4.

2.3.4 Capillary GC analysis of FAME's

The purity of FAME's were determined with a Carlo Erba HRGC 5300 mega series flame ionization gas chromatograph. The conditions employed were: column; length 50m, inside diameter 0.32 mm, outside diameter 0.45 mm, wall coated open tubular (WCOT) with CP Sil 88 liquid phase (film thickness 0.37mm); isocratic run - column temp. 195 °C, injector & detector temp. 250 °C, carrier gas - helium; bottom split 80 ml/min., top split 3 ml/min., split ratio was approximately 50:1. Approximately 1 μl of the FAME solution (concentration 20 g/l) was injected into the GC system. Quantitation was achieved by means of the automatic electronic integrator of the Perkin Elmer sigma 10 series.

The peaks on the chromatograms obtained in this thesis were identified by direct comparison of the retention times with those of a standard mixture. This standard mixture was fully characterised for the CP Sil-88 column by the MAFF. Furthermore, the composition of FAME's in this standard mixture resembled that expected in hydrogenated vegetable oils.
2.3.5 Storage of FAME's

The FAME's arrive from the manufacturer in sealed vials which had been purged with dry nitrogen before sealing to prevent them from reacting with oxygen and water. Some of the more exotic FAME's are also light sensitive and are supplied in amber vials. All of the FAME's are heat sensitive and were kept in a freezer as recommended by the manufacturer. An exception was nervonic acid methyl ester, which was stored between 0 °C and 5 °C in a refrigerator. Once the vials were opened they were stored in a vacuum desiccator which had been evacuated and filled with dry nitrogen several times before being placed in a freezer. The vacuum desiccator was taken out of the freezer and allowed to reach room temperature in a dark cupboard before samples were removed.

2.3.6 Preparation of deuterated methyl elaidate

Deuterated methyl elaidate (CH₃-(CH₂)₇-CH=CH-(CH₂)₇-CO₂-CD₃) was prepared according to the procedure described by Nolin (1954). This involved the preparation of silver elaidate by adding at the same rate equivalent amounts of silver nitrate (1.2g in 250ml) and potassium elaidate (2.27g in 500ml of 50/50 ethanol:water warmed) to hot water with stirring. The precipitate was filtered off, washed with water and then acetone, and dried under vacuum in the dark. One gram of dry silver elaidate was placed in a tube (2 cm x 15 cm). After the tube was constricted to facilitate subsequent sealing, 4ml of anhydrous ether and 0.48g of methyl-d₃ iodide were placed into the tube, which was sealed with a suber seal. The tube was frozen in liquid nitrogen, evacuated, and sealed by closing constriction of the tube. The reactants were heated at 65°C in an oil bath while rocking (covered with aluminium foil) for 20 hours. Silver iodide was filtered off and washed with anhydrous ether. The ether fraction was washed with H₂O several times then activated charcoal added (0.5g). The charcoal was filtered off and the ether was evaporated off under vacuum.

The purity and percentage deuteration of the methyl-d₃-elaidate prepared above were established by GC and NMR, respectively. The GC chromatogram showed only one band with a retention time of 345 seconds (methyl elaidate 339 sec. and methyl oleate 352 sec.). NMR bands corresponding to the protons on the carbomethoxy group were absent, indicating > 99% deuteration of this group.
2.3.7 Preparation of methyl-d₃-stearate

The methyl-d₃-stearate (CH₃-(CH₂)₁₆·C₀₂·CD₃) was prepared by hydrogenating methyl-d₃-elaidate (100mg) using platinum dioxide catalyst as described by Hirabayashi et al. (1971). The hydrogenation was followed by GC. Once all the methyl-d₃-elaidate had been converted to methyl-d₃-stearate the reaction was stopped and the catalyst filtered off before the solvent was removed by distillation under reduced pressure. The remaining yellow solid was recrystallized from a mixture of ethanol and water (50/50). The melting point of the recrystallized methyl-d₃-stearate was 33.5 to 34.5°C, which is lower than that quoted for methyl stearate (38.5-38.9°C - Markley, 1960).

2.3.8 Spectral enhancement (deconvolution)

The enhancement technique, also called deconvolution, was used to resolve overlapping spectral bands. The way in which the ENHANCE program operates is most easily explained by starting with Fourier deconvolution, as described by Kauppinen et al. (1981a).

The Fourier deconvolution method works on the principle that the Lorentzian profile (i.e. spectral band, see figure 2.3.8.1) yields a sinusoidal interferogram with exponential envelopes. The narrower the width of the spectral band, the greater the width of the envelope of the interferogram (figures 2.3.8.1 & 2.3.8.2). Conversely, for broad bands spectral sources, the decay is very rapid. Thus by multiplying by a function which elongates the interferogram and converting back to the spectrum, bands which overlap are made narrower and hence resolved (figure 2.3.8.3). The function used is of the form shown in equation 2.3.8.1, where a can be varied to modify the effect of the function.

Eqn. 2.3.8.1

\[ Y = e^{ax} \]

However, this operation may introduce negative side lobes to the bands, and this is overcome by multiplying by an apodization function of the type shown in equation 2.3.8.2, where b is a variable.

Eqn. 2.3.8.2

\[ Y = \cos^4(bx) \]
The ENHANCE program does the equivalent of the Fourier deconvolution by an operation which acts directly on the frequency spectrum. It is a variation of a smoothing function, which replaces each point in the spectrum with a weighted average of neighbouring points. The nett effect is that the intensity from the wings of a band are transferred to its centre. There are two variables (width and factor) in the ENHANCE program which can be used to change the a and b variables in the equations above. The best values for the two variables were established empirically on the spectrum by achieving the best separation between the overlapping bands but with the minimum "ripple". This technique is generally used to resolve overlapping bands (Lipp & Nafie, 1984; Yang & Griffiths, 1981) but has also been used quantitatively by Willis et al. (1984) to determine the length of the methylene sequence in ethylene propylene copolymers, in order to establish their physical and chemical properties.

2.3.9 Preparation of linoleic acid

Linoleic acid was prepared from sunflower oil (200g) according to the method described by McCutcheon (1942). Briefly, this involved the alkali hydrolysis of the triglycerides to the free fatty acids, which were separated by filtration after the addition of water and acidification of the solution. The fatty acids were dissolved in petroleum ether and then brominated to form the tetrabromostearic acid. This product was purified by recrystallizing in petroleum ether followed by ethylene dichloride and finally washing with petroleum ether. The quantities recommended in this method were doubled and the methyl ester was prepared instead of the ethyl ester of linoleic acid in the final purification stage, in order to simplify the GC and IR analysis of the product. The modification to the esterification stage, which also involved changes to the debromination, were as follows; in to a 500ml Erlenmeyer flask were placed the recrystallized tetrabromostearic acid (60g), methanol (200ml) and zinc powder (60g). This mixture was warmed gently in order to initialise the debromination. This reaction was extremely exothermic and needed to be moderated by immersing the flask in cold water at regular intervals. After the vigorous reaction had subsided, the flask was fitted with a reflux condenser and the mixture refluxed for half an hour. In order to esterify the linoleic acid so formed, 20ml of a 4N solution of hydrochloric acid in methanol was poured into the refluxing mixture through the top of the condenser. Four successive 10ml aliquots of HCl were added at thirty minute intervals. At the end of this time the solution was removed from the unchanged zinc by decanting into another flask. The zinc was washed with
30ml of methanol to complete the transfer of the solution. A 20ml aliquot of 4N methanolic hydrochloric acid was then added and the solution refluxed for an hour, with addition of a second aliquot of methanolic hydrochloric acid (20ml) after thirty minutes. The mixture was poured into a 1L separating funnel containing a hot saturated NaCl solution (600ml), and the ester allowed to separate for half an hour. The aqueous layer was removed, and the ester washed at room temperature with 0.5% (w/v) sodium carbonate solution (600ml).

Table 2.3.10

Showing the boiling point and composition of each fraction of the distilled conjugated and unconjugated isomers of methyl linoleate.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Boiling point (°C)</th>
<th>Composition determined by GC (% of total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>128 (0.135mmHg)</td>
<td>18:2-90.1%, b-4.2%, c-5.7%, d &lt; 0.1%</td>
</tr>
<tr>
<td>2</td>
<td>130 (0.135mmHg)</td>
<td>18:2-84.5%, b-7.1%, c-8.4%, d &lt; 0.1%</td>
</tr>
<tr>
<td>3</td>
<td>134-136 (0.135mmHg)</td>
<td>18:2-79.3%, b-10.4%, c-10.3%, d &lt; 0.1%</td>
</tr>
<tr>
<td>4</td>
<td>128 (0.11mmHg)</td>
<td>18:2-69.9%, a &lt; 0.1%, b-15.4%, c-14.3%, d-0.3%</td>
</tr>
<tr>
<td>5</td>
<td>128-129 (0.11mmHg)</td>
<td>18:2-42.2%, a &lt; 0.1%, b-22.4%, c-34.8%, d-0.5%</td>
</tr>
<tr>
<td>6</td>
<td>135-137 (0.11mmHg)</td>
<td>18:2-13.7%, a-0.2%, b/c-82.9%, d-3.2%</td>
</tr>
<tr>
<td>7</td>
<td>137-140 (0.11mmHg)</td>
<td>18:2-3.3%, a-0.1%, b-28.1%, c-66.3%, d-2.2%</td>
</tr>
</tbody>
</table>

Abbreviations used on Table 2.3.10; 18:2 = methyl linoleate
a, b, c, d represent the peaks shown in figure 2.3.10 which are unidentified FAME's produced during the isomerisation of methyl linoleate.
The stable emulsion described by McCutcheon in the paper did not form, so the section on centrifuging was missed. Instead the two layers were allowed to separate over night before being separated, and the ester layer was washed repeated with warm water until the wash water was neutral. The neutral ester was then transferred to a distillation flask and distilled under reduced pressure; boiling point 156°C/0-16 torr (10% yield).

The product was a clear liquid with an IR spectrum almost identical to that of a methyl linoleate standard (purchased from Sigma), except for the presence of a small band at 969 cm⁻¹ which suggests the presence of some of the trans isomer. The purity of the product was found to be 96.87% by GC. The GC chromatogram showed the presence of a small peak just in front of the methyl linoleate peak, possibly trans-trans or cis-trans isomers.

2.3.10 Preparation of mixed isomers of methyl linoleate

A mixture of conjugated and unconjugated isomers of methyl linoleate were prepared by alkaline-isomerisation of linoleic acid (20g), prepared in Section 2.3.9, according to the method described by Nichols et al. (1951). The isomerised acids were converted to the methyl esters using the procedure described in Section 2.3.9 and then subjected to careful fractional distillation under reduced pressure (N₂ atmosphere). Table 2.3.10 shows the boiling point of each fraction collected and the composition determined by GC.

2.3.11 Semiempirical molecular orbital calculations

The AMPAC semiempirical molecular orbital program was used to calculate the vibrational frequencies of the simple unsaturated molecules listed in Table 2.4.3.3. It is beyond the scope of this thesis to explain the complex programs/mathematical equations used and therefore only a brief overview will be presented. A first class study by Stewart (1990) describes the details of the semiempirical calculations using MOPAC the principles of which are similar to the AMPAC program.

Each of the molecular structures required were drawn using ChemX in the appropriate
conformations and the information transferred to the AMPAC package as the MNDO where the parameters listed in Table 2.3.11 were entered for each of the corresponding molecules. After calculating the force constants, it is straightforward to mass-weight the force matrix and calculate the vibrational frequencies. Vibrational mode results obtained include the frequency and those pair of atoms which are most strongly involved in each vibration.

Table 2.3.11

Parameters used in AMPAC program where; molecule designates the structure (see Tables 2.4.3.3 & 2.4.3.4), c & t are cis and trans, Rot - symmetry number specified, Force - force calculation specified, thermo - the thermodynamic quantities to be calculated.

<table>
<thead>
<tr>
<th>Molecule</th>
<th>no. of CH=CH</th>
<th>type</th>
<th>Rot</th>
<th>parameters specified</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>1</td>
<td>c</td>
<td>2</td>
<td>Force/thermo</td>
</tr>
<tr>
<td>B</td>
<td>1</td>
<td>t</td>
<td>2</td>
<td>Force/thermo</td>
</tr>
<tr>
<td>C</td>
<td>2</td>
<td>c, c</td>
<td>2</td>
<td>Force/thermo</td>
</tr>
<tr>
<td>D^X</td>
<td>3</td>
<td>c, c, c</td>
<td>2</td>
<td>Force/thermo</td>
</tr>
<tr>
<td>D^Y</td>
<td>3</td>
<td>c, c, c</td>
<td>2</td>
<td>Force/thermo</td>
</tr>
<tr>
<td>D^Z</td>
<td>3</td>
<td>c, c, c</td>
<td>2</td>
<td>Force/thermo</td>
</tr>
<tr>
<td>E</td>
<td>3</td>
<td>t, t, t</td>
<td>2</td>
<td>Force/thermo</td>
</tr>
</tbody>
</table>

2.4 RESULTS AND DISCUSSION

2.4.1 Comparison of the "normal" and enhanced spectra of various unsaturated cis and trans FAME's

In order to capitalize fully on the added advantages that FTIR offers over conventional dispersive IR instruments, a program of investigations was carried out in which the various unsaturated and saturated FAME's spectra in solution and the liquid phase were compared to establish any
spectral differences. To simplify the task of interpreting the results, the spectral region studied (4000 to 400 cm⁻¹) was subdivided into the following sections:

Region 1 - 4000 to 2600 cm⁻¹
Region 2 - 2600 to 1600 cm⁻¹
Region 3 - 1600 to 1000 cm⁻¹
Region 4 - 1000 to 600 cm⁻¹

In some cases these sections are divided further to describe dissimilarities arising due to either the number of double bonds, the number of α methylenes adjacent to the double bond(s), the number of single methylenes lying between the double bonds (referred to as β methylenes) in poly-unsaturated FAME's, and finally, the number of methylenes in the chains attached to either the terminal methyl group (m) or the carboxylic group (n) (see figure 2.4.1). In addition to the comparative studies on the "normal" spectra of the various unsaturated and saturated FAME's, data provided by the deconvoluted and "difference" spectra was used. These two techniques helped to enhance spectral differences.

2.4.2 Region 1 - 4000 to 2600 cm⁻¹

2.4.2.1 Spectral differences in region 1 between different cis mono-unsaturated and saturated FAME's

No major spectral differences were observed between the various cis mono-unsaturated FAME's listed in Table 2.4.2.1, apart from the intensities of the bands ascribed to the methylene vibrations which increased with prolongation of the methylene chains on either side of the double bond. Figures 2.4.2.1.1 and 2.4.2.1.2 show the "normal" and enhanced spectra of methyl oleate (18:1a9c) which is typical for this series of FAME's. The only spectral difference observed between the "normal" spectra of methyl oleate and methyl stearate, representing a typical saturated FAME (figure 2.4.2.1.3), is the cis double bond carbon hydrogen stretch at 3005 cm⁻¹ which is well documented in the literature.

Before the "difference" spectrum is discussed an explanation of the interpretation of this type of spectrum is considered appropriate. In the case of figure 2.4.2.1.4 which shows the "difference" spectrum obtained by the interactive subtraction of the spectrum of methyl stearate from that of
methyl oleate, positive and negative peaks were observed. Those bands which are positive correspond to bands which are either of greater intensity or are only present in the spectrum of methyl oleate. Negative bands are therefore bands of either greater intensity or only present in the methyl stearate spectrum.

Table 2.4.2.1: Shows the cis mono-unsaturated FAME's studied and the number of methylenes in each FAME on either side of the double bond, where m and n are:

\[
\text{CH}_3\text{-(CH}_2\text{)}_m\text{-CH=CH-(CH}_2\text{)}_n\text{-CO}_2\text{-CH}_3
\]

<table>
<thead>
<tr>
<th>Cis FAME's</th>
<th>Number of methylenes on chains</th>
<th>Spectrum no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:1\Delta9c</td>
<td>3 7</td>
<td>SP004</td>
</tr>
<tr>
<td>16:1\Delta9c</td>
<td>5 7</td>
<td>SP006</td>
</tr>
<tr>
<td>18:1\Delta6c</td>
<td>10 4</td>
<td>SP008</td>
</tr>
<tr>
<td>18:1\Delta9c</td>
<td>7 7</td>
<td>SP010</td>
</tr>
<tr>
<td>18:1\Delta11c</td>
<td>5 9</td>
<td>SP012</td>
</tr>
<tr>
<td>18:1\Delta9c12(OH)</td>
<td>5 7</td>
<td>SP014</td>
</tr>
<tr>
<td>19:1\Delta10c</td>
<td>7 8</td>
<td>SP020</td>
</tr>
<tr>
<td>20:1\Delta8c</td>
<td>10 6</td>
<td>SP021</td>
</tr>
<tr>
<td>20:1\Delta11c</td>
<td>7 9</td>
<td>SP022</td>
</tr>
<tr>
<td>22:1\Delta13c</td>
<td>7 11</td>
<td>SP028</td>
</tr>
<tr>
<td>24:1\Delta15c</td>
<td>8 13</td>
<td>SP031</td>
</tr>
</tbody>
</table>

From the above description the two large negative bands at 2925 cm\(^{-1}\) and 2854 cm\(^{-1}\) observed in figure 2.4.2.1.4 can be attributed to the methylene stretching vibrations, which are of greater intensity in the spectrum of methyl stearate than in methyl oleate, due to the presence of two extra methylenes. Similarly, the cis double bond carbon hydrogen stretch, which occurs only in the spectrum of methyl oleate, is observed as a positive band in the difference spectrum. It was interesting to note a small positive band at approximately 2842 cm\(^{-1}\) (band g) in the "difference" spectrum which corresponded with a band in the enhanced spectrum of methyl oleate (figure 2.4.2.1.2). This band was not present in the enhanced spectrum of methyl stearate and must
therefore be associated with either the double bond or with the adjacent methylenes. The enhanced spectrum of methyl oleate also revealed an inflection (band $\beta$) at the higher wavenumber side of the terminal methyl asymmetrical carbon hydrogen stretch band (band c). This band is not observed in the "normal" spectrum. Examination of the enhanced methyl stearate spectrum also revealed this inflection which suggests that it is the normally concealed methoxy symmetrical carbon hydrogen stretch (-CO$_2$-CH$_3$, see Section 2.2.1 Table 2.2.1.1). Further conformation was provided by the "difference" spectrum which showed no positive or negative bands in this region.

2.4.2.2 Spectral differences in region 1 between various cis poly-unsaturated FAME's

In this section, the "normal" and deconvoluted liquid phase spectra of the various cis poly-unsaturated FAME's listed in Table 2.4.2.2 were studied. The "normal" spectra of the cis di-unsaturated FAME's (18:2$\Delta$9c12c & 20:2$\Delta$11c14c) were very similar to those of the mono-unsaturated FAME's, except for the relative intensities of the cis =C-H stretch and methylene stretching bands as expected. Figure 2.4.2.2.1 shows the spectrum of methyl linoleate (18:2$\Delta$9c12c) in this region and is representative of the di-unsaturated FAME's. No spectral differences were observed between the deconvoluted spectra of the two di-unsaturated FAME's. Differences were observed between the deconvoluted spectra of di- and mono-unsaturated FAME's. For example, a band at 2991 cm$^{-1}$ (band b) was observed only in the di-unsaturated enhanced spectra, whereas band g at 2842 cm$^{-1}$, observed in the spectra of mono-unsaturated FAME'S was not as prominent (see figure 2.4.2.2.2).

The deconvoluted solution phase spectrum of methyl linoleate (figure 2.4.2.2.3) was unexpectedly found to have the spectral bands far more enhanced than the corresponding liquid phase spectrum (figure 2.4.2.2.2). This was surprising because it suggests that there are a greater number of conformations in the liquid phase over the solution. As the degree of freedom of the molecule increases then the bands become broader and hence the greater the overlap between them. Bands therefore become progressively more difficult to resolve.

The deconvoluted solution spectrum of methyl linoleate revealed the presence of band g, unobserved in the corresponding deconvoluted liquid phase spectrum, as well as band b. In addition, the $\beta$ band was clearly resolved from that of band c. Examination of the deconvoluted
solution spectra of cis mono-unsaturated FAME's (represented by methyl vaccenate figure 2.4.2.2.4) also showed better spectral resolution than the corresponding deconvoluted liquid phase spectra. However, the \( \beta \) band still occurred as a slight inflection. This implied that the \( \beta \) band in the di-unsaturated FAME's comprises of more than one band because the intensity of this band (\(-\text{CO}_2\cdot\text{CH}_3\) sym. C-H stretch) should remain constant relative to band c (\(-\text{CH}_3\) asym. C-H stretch) in both the di- and mono-unsaturated FAME spectra. However, this band was found to increase with the number of cis isolated double bonds. The band or bands which overlap with band \( \beta \) will be referred to as band \( \beta' \) in the following passages.

Band \( \beta' \) must be associated with one of the following group vibrations: the double bond, the methylenes adjacent to the double bonds but connected to the methylene chains (\( \alpha \) methylenes), the methylene lying between the two double bonds (\( \beta \) methylene) or all the methylenes adjacent to the double bonds (\( \alpha + \beta \) methylenes, see Table 2.4.2.2).

Of the four tri-unsaturated cis FAME's studied, two (18:3\( \Delta \delta c9c12c & 20:3\Delta \delta c11c14c \)) had "normal" spectra in this region with similar spectral features to those of the mono- and di-unsaturated FAME's studied (see figure 2.4.2.2.5). The deconvoluted solution and liquid phase spectra of these two tri-unsaturated FAME's were similar to those of the corresponding di-unsaturated FAME's (see figures 2.4.2.2.6 and 2.4.2.2.7). The other two tri-unsaturated cis FAME's studied (18:3\( \Delta \delta c12c15c & 20:3\Delta \delta c14c17c \)) had one major spectral difference which distinguishes them from the other two tri-unsaturated cis FAME's discussed above. This difference is best observed in the deconvoluted solution phase spectrum (see figure 2.4.2.2.8) which shows the increased intensity of the \( \beta' \) band and a shift to higher frequency, from 2961 cm\(^{-1}\) in the other tri-FAME's to 2965 cm\(^{-1}\). Once again this spectral difference is not apparent in the "normal" spectra. The most significant difference between these two spectrally different sets of cis tri-unsaturated FAME's is that the latter group, in which the \( \beta' \) band is prominent, have only one methylene between the double bond and the terminal methyl group (m=1), whereas in the other set of FAME's m > 2.

Two poly-unsaturated cis FAME's containing four unconjugated double bonds were studied. One of these FAME's (22:4\( \Delta \gamma c7c10c13c16c \)) had "normal" and enhanced spectra which resembled those of the mono-, di- and tri-unsaturated FAME's with m > 2 (see figure 2.4.2.2.9). Band \( \beta + \beta' \) were unresolved from that of band c in the spectrum of this FAME. The intensity of band \( \beta' \) though, appeared to be greater than in the spectra of the other poly-unsaturated FAME's. The other tetra-unsaturated FAME studied (18:4\( \Delta \delta c9c12c15c \)) contained only one methylene between the terminal methyl group and the double bond and as expected, the normal and
enhanced spectra resembled those of the tri-unsaturated FAME's with m = 1. Figure 2.4.2.2.10 shows the deconvoluted solution phase spectrum of this FAME, in which the bands associated with the methylene chains (bands d and f) are greatly reduced in intensity allowing bands obscured in other FAME's, with larger numbers of methylenes, to be observed. One such region, which is indicated by an X in figure 2.4.2.2.10, shows a number of overlapping bands. These bands have been observed in the deconvoluted spectra of other FAME's which occur as small inflections on the lower wavenumber side of band d. Bands in region X have not been reported in the literature cited. Band g (2843 cm⁻¹) was also better resolved due to the decrease in intensity of the methylene chain vibrations (band f).

The highest poly-unsaturated FAME to be studied was methyl docosahexenoate (22:6Δ4c7c10c13c16c19c). This FAME contained only two methylenes on the n chain and one on the m methylene chain and hence the deconvoluted solution spectrum (figure 2.4.2.2.11) resembles that of 18:4Δ6c9c12c15c (figure 2.4.2.2.10). Band β + β' in both the above mentioned spectra could clearly be seen to be a combination of at least two bands.

**Table 2.4.2.2**

Shows the cis poly-unsaturated FAME's studied and the number of methylenes in each FAME on either side of the double bond, where m and n are:

\[
\text{Cis FAME's} \quad \text{Number of methylenes on} \quad \text{Spectrum no.}
\]

<table>
<thead>
<tr>
<th>Cis FAME's</th>
<th>Number of methylenes on chains</th>
<th>Spectrum no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2Δ9c12c</td>
<td>4, 7</td>
<td>SP016</td>
</tr>
<tr>
<td>20:2Δ11c14c</td>
<td>4, 9</td>
<td>SP024</td>
</tr>
<tr>
<td>18:3Δ6c9c12c</td>
<td>4, 4</td>
<td>SP018</td>
</tr>
<tr>
<td>18:3Δ9c12c15c</td>
<td>1, 7</td>
<td>SP019</td>
</tr>
<tr>
<td>20:3Δ8c11c14c</td>
<td>4, 6</td>
<td>SP026</td>
</tr>
<tr>
<td>20:3Δ11c14c17c</td>
<td>4, 4</td>
<td>SP027</td>
</tr>
<tr>
<td>18:4Δ6c9c12c15c</td>
<td>1, 4</td>
<td>*</td>
</tr>
<tr>
<td>22:4Δ7c10c13c16c</td>
<td>4, 5</td>
<td>SP030</td>
</tr>
<tr>
<td>22:6Δ4c7c10c13c16c19c</td>
<td>1, 2</td>
<td>*</td>
</tr>
</tbody>
</table>

Where - * = Used solution phase spectra
Spectral studies were conducted on the trans FAME's listed in Table 2.4.2.3. No spectral differences were observed between the mono-unsaturated trans FAME's studied, apart from the intensities of the bands associated with the methylene chains which increased with increase in chain length as expected (relative to the carbonyl band which remains constant, see section 4.4.8.3).

Table 2.4.2.3

Shows the trans unsaturated FAME's studied and the number of methylenes in each FAME on either side of the double bond, where m and n are:

\[
\text{CH}_3-(\text{CH}_2)_m\text{CH=CH-(CH}_2\text{-CH=CH}_2\text{)}_n\text{-CO}_2\text{-CH}_3.
\]

<table>
<thead>
<tr>
<th>Trans FAME's</th>
<th>Number of methylenes on chains</th>
<th>Spectrum no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:1Δ9t</td>
<td>m 3 n 7</td>
<td>SP005</td>
</tr>
<tr>
<td>16:1Δ9t</td>
<td>5 7</td>
<td>SP007</td>
</tr>
<tr>
<td>18:1Δ6t</td>
<td>10 4</td>
<td>SP009</td>
</tr>
<tr>
<td>18:1Δ9t</td>
<td>7 7</td>
<td>SP011</td>
</tr>
<tr>
<td>18:1Δ11t</td>
<td>5 9</td>
<td>SP013</td>
</tr>
<tr>
<td>18:1Δ9t12(OH)</td>
<td>5 7</td>
<td>SP015</td>
</tr>
<tr>
<td>20:1Δ11t</td>
<td>7 9</td>
<td>SP023</td>
</tr>
<tr>
<td>22:1Δ13t</td>
<td>7 11</td>
<td>SP029</td>
</tr>
<tr>
<td>18:2Δ9t12t</td>
<td>4 7</td>
<td>SP017</td>
</tr>
<tr>
<td>20:2Δ11t14t</td>
<td>4 9</td>
<td>SP025</td>
</tr>
</tbody>
</table>

The "normal" and deconvoluted spectra of methyl elaidate, which are representative of corresponding trans mono-unsaturated FAME's spectra, are shown in figures 2.4.2.3.1 and 2.4.2.3.2, respectively. These figures also show the assignments of the various known bands.
Comparison of the enhanced spectra of the representative cis and trans mono-unsaturated FAME's revealed (methyl elaidate and methyl oleate, figures 2.4.2.3.2 and 2.4.2.1.2) no significant differences, apart from the noticeable absence of the cis -C=H stretch in the latter. The absence of the cis =C-H stretch allows two bands at 3026 cm\(^{-1}\) and 2990 cm\(^{-1}\) to be observed in the spectrum of methyl elaidate. They are also present in the spectrum of methyl stearate, and are assumed to be obscured in the methyl oleate spectrum by the overlapping cis =C-H stretch.

Wilmshurst (1957) has assigned the bands at 3026 cm\(^{-1}\) and 2999 cm\(^{-1}\) of methyl acetate to asymmetrical C-H stretching modes of the methoxyl group, assuming that the molecule has the planar s-cis conformation shown in figure 2.4.2.3.3. The normally doubly degenerate mode here is split due to the presence of a plane of symmetry. The 3026 cm\(^{-1}\) band is attributed to the a' species symmetry with respect to the plane, and the 2999 cm\(^{-1}\) band to the a'' antisymmetrical species. The presence of a similar doublet in the spectra of methyl laurate (Jones, 1962) and methyl stearate (figure 2.4.2.1.3) but not in the deuterated methyl laurate ester indicates that the carbomethoxyl group has the same s-cis conformation. Wilmshurst's evidence for the s-cis conformation in methyl acetate is not conclusive, but it is based on analogy with methyl formate. Wilmshurst assigned the band at \(\approx 2957\) cm\(^{-1}\) in the spectrum of methyl acetate to the symmetrical C-H stretching mode of the methoxyl group. This band was not observed in the spectra of FAME's, unless they were enhanced, due to overlap with the band at \(\approx 2950\) cm\(^{-1}\), attributed to the methyl group (asymmetrical C-H stretching mode) at the end of the methylene chain.

Spectral studies were also conducted on di-unsaturated trans FAME's (18:2\(\Delta9t12t\) and 20:2\(\Delta11t14t\)) which were found to have similar spectral features to the mono-trans (figures 2.4.2.3.4 and 2.4.2.3.5). Spectral differences between the di- and mono-unsaturated trans FAME's were only apparent in the enhanced spectra and these differences were limited to increases in the intensities of certain bands and the appearance of an additional small band which occurred as an inflection at 2828 cm\(^{-1}\) in the di-unsaturated FAME's (band h in figure 2.4.2.3.5). Bands associated with the differences in intensities are listed below with their corresponding positions in the enhanced spectra:

- Band A - 3028 cm\(^{-1}\)
- Band B - 2991 cm\(^{-1}\)
- Band d - 2922 cm\(^{-1}\)
- Band f - 2852 cm\(^{-1}\)
Quantitative solution phase studies have confirmed that the intensities of bands A and B increase with the number of trans double bonds (see section 3.4.3). This suggests that bands attributed to the trans double bond also absorb in these positions. Further evidence to support the above proposition was provided by the spectrum of methyl-d₃-elaidate, in which the methoxyl bands A and B are shifted to lower frequencies due to the isotope effect. The spectrum still showed the presence of two bands at ~3028 cm⁻¹ and 2980 cm⁻¹ (called B' and B'⁰). The latter of these bands is very weak and easily overlooked. Jones (1962) also showed that the methoxyl bands A and B were shifted to lower frequencies in the spectrum of methyl-d₃-laurate. To ensure that bands B' and B'⁰ observed in the spectrum of methyl-d₃-elaidate corresponded to the trans double bond, the spectrum of methyl-d₃-stearate was studied. No spectral bands were observed in the region in question (see figure 2.4.2.3.6). This provides yet more evidence to support the assignment of bands B' and B'⁰ to trans double bond vibrations. The nature of these vibrations is unclear.

The most obvious modes of vibration for the trans bands at 3028 cm⁻¹ and 2980 cm⁻¹ were the asymmetrical and symmetrical =C-H stretching vibrations across the double bond as shown in figure 2.4.2.3.7. A more detailed study of the literature disclosed that bands attributed to the trans =C-H stretching vibrations had been observed in the spectrum of trans-butene at 3021 cm⁻¹ in the IR and 3007 cm⁻¹ in the Raman (Sheppard & Simpson, 1952; Sverdlov et al., 1974). However, Hirabayashi et al. (1971) observed that the =C-D stretching vibration of deuterated ethylenic double bonds (-CD=CD-) in methyl-9,10-dideuter-oleate and methyl-9,10- dideuter-olaidate occurred at 2250 and 2225 cm⁻¹ respectively. This observation suggests that both the cis and trans =C-H stretching vibrations have only one absorption band in the IR, namely the band at ~3025 cm⁻¹. The other trans band at 2980 cm⁻¹ could be due to the C-H stretch of the alpha methylene groups adjacent to the ethylenic double bond. There is, however, the distinct possibility that the latter band may have been overlooked by Hirabayashi et al. in the deuterated compounds, so no definite conclusion can be reached about the assignment of this band.

Figure 2.4.2.3.8 shows the "difference" spectrum of methyl elaidate minus methyl stearate. The interpretation of this type of spectrum has been described in Section 2.4.2.2. Key points with respect to this spectrum are that three positive peaks were observed, which correspond to bands which are of either higher intensity in the spectrum of methyl elaidate or not present in the spectrum of methyl stearate. These bands are indicated on the spectrum and mirror those differences described between the deconvoluted spectra of trans and saturated FAME's.
**2.4.2.4 Spectral differences in region 1 between methyl linoleate and the various conjugated isomer fractions**

Figure 2.4.2.4 shows the spectra of the 4th, 5th and 6th distillation fractions obtained from the alkaline isomerisation of methyl linoleate in the region 3100 to 2970 cm⁻¹. The band associated with the di-cis isolated double bond =C-H stretching vibration at 3011 cm⁻¹ was seen to decrease in intensity as the concentration of methyl linoleate in the fractions studied diminished. The bands at 3020 cm⁻¹ and 3005 cm⁻¹ increase in intensity with increasing conjugated isomer content. It is interesting to note that the new bands appear to absorb close to where the mono-cis and mono-trans isolated bond C-H stretch would be expected; 3005 cm⁻¹ and 3025 cm⁻¹ respectively.

**2.4.3 Region 2 - 2600 to 1600 cm⁻¹**

All the unsaturated and saturated spectra of the FAME's had a common spectral band at ≈ 1742 cm⁻¹ attributed to the methoxyl carbonyl stretch (C=O). Another spectral feature shared by all the FAME's was a weakly absorbing inflection on the lower wavenumber side of the carbonyl band. The assignment of this band is not known.

The C=C stretching vibration was observed in the spectra of all the cis unsaturated FAME's. The differences in profile of this band which occur with changes in the number of methylene interrupted cis double bonds are of interest (Figure 2.4.3.1). Spectral enhancement of these bands revealed that one band was present for mono-, two overlapping bands for di- and three overlapping bands for tri-unsaturated cis FAME's (see figures 2.4.3.2, 2.4.3.3 and 2.4.3.4).

Table 2.4.3.1 shows the positions of the cis C=C stretching bands observed in the normal and enhanced solution spectra of the various cis FAME's containing different numbers of double bonds. Differences were apparent in the enhanced spectra of the various cis FAME studied containing three isolated double bonds. This is illustrated by figures 2.4.3.4 and 2.4.3.5, which represent the spectra of 18:3Δ6c9c12c and 18:3Δ9c12c15c. Here the former shows the intensity of bands a and b (indicated on spectrum) to be of similar intensity and band a to be broad and unsymmetrical due to the presence of an unresolved band (called band c) that absorbs at a
slightly higher frequency. In the case of the latter spectrum, band a is of greater intensity than that of b due to the shift of the overlapping band (just apparent as a slight inflection) to a lower frequency.

Table 2.4.3.1; shows the positions of the cis C=C stretching bands in cis FAME's contain different numbers of isolated double bonds observed in normal and enhanced spectra, where; m indicates the number of methylenes present between the terminal methyl and double bonds; ub = unsymmetrical broad band; sh = shoulder observed on higher frequency side; b = broad band; d = difference between resolved bands.

<table>
<thead>
<tr>
<th>Class of cis FAME</th>
<th>Position of cis C=C stretching band(s) in cm⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ordinary</td>
</tr>
<tr>
<td>mono-</td>
<td>1654</td>
</tr>
<tr>
<td>di-</td>
<td>1657 (d 9)</td>
</tr>
<tr>
<td>tri- (m&gt;1)</td>
<td>1653 (ub)</td>
</tr>
<tr>
<td>tri- (m=1)</td>
<td>1653 (ub)</td>
</tr>
<tr>
<td>tetra- (m&gt;1)</td>
<td>1655 (ub)</td>
</tr>
<tr>
<td>tetra- (m=1)</td>
<td>1654 (ub)</td>
</tr>
<tr>
<td>hex-</td>
<td>1654 (ub)</td>
</tr>
</tbody>
</table>

These dissimilarities are considered to be attributable to differences in the number of methylenes on either side of the double bonds as shown by Table 2.4.3.2. Further studies on other cis isolated tri-unsaturated FAME's (indicated on Table 2.4.3.2) supported this explanation. Band c occurred at a lower frequency when only one methylene was present between the double bond and the terminal methyl group (m = 1). Increasing the number of methylenes (n) between the
carbonyl and the double bonds had little effect upon the positions of the C=C stretching bands unless, of course, \( n = 1 \) when conjugation will occur with the carbonyl group.

Table 2.4.3.2 shows the influence of the methylene chains on either side of the double bonds upon the positions of the cis C=C stretching bands. Enhanced spectra types I, II, III and IV resemble figures 2.4.3.4, 2.4.3.5, 2.4.3.6 and 2.4.3.7 respectively. The numbers \( m \) and \( n \) correspond to \( \text{CH}_3-(	ext{CH}_2)_m-	ext{CH}=	ext{CH}-(	ext{CH}_2-	ext{CH}=	ext{CH})_z-(	ext{CH}_2)_n-	ext{CO}_2\text{CH}_3 \).

<table>
<thead>
<tr>
<th>Cis FAME's</th>
<th>Number of methylenes on either side chains</th>
<th>Type of spectrum</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( m )</td>
<td>( n )</td>
</tr>
<tr>
<td>18:3Δ6c9c12c</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>18:3Δ9c12c15c</td>
<td>1</td>
<td>7</td>
</tr>
<tr>
<td>20:3Δ8c11c14c</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>20:3Δ11c14c17c</td>
<td>1</td>
<td>9</td>
</tr>
<tr>
<td>18:4Δ6c9c12c15c</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td>20:4Δ5c8c11c14c</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>22:4Δ7c10c13c16c</td>
<td>4</td>
<td>5</td>
</tr>
</tbody>
</table>

Similar effects to those described in the previous paragraph were observed in the enhanced spectra of the FAME's containing four isolated double bonds. Those FAME's having more than one methylene between the double bonds, and the terminal methyl group, were observed to have three distinct bands and an inflection on the higher frequency band, suggesting the overlap of a smaller band (see figure 2.4.3.7). In the case of 18:4Δ6c9c12c15c (figure 2.4.3.6), which contains only one methylene in the \( m \) chain, only two bands were resolved. The peak position of the highest frequency band (band a) occurs at a lower frequency than that of the other tetra-ene FAME where \( m > 1 \). No spectral differences were apparent between the ordinary spectra of any of the FAME's containing four isolated double bonds. Figure 2.4.3.8 represents the enhanced spectrum of 22:6Δ4c7c10c13c16c19c in which at least two unresolved bands were observed.

The above results suggest that the spectrum of a FAME containing one isolated cis double bond has one band corresponding to the C=C stretch, two isolated cis double bonds two bands, three
cis double bonds three bands and so forth. These bands occur in a well defined spectral window (1670 to 1630 cm\(^{-1}\)), thus little or no resolution between the bands is observed when more than four isolated cis bands are present. The results contradict the accepted conclusion that double bonds separated by one or more methylenes hardly interact. This conclusion was based on mode calculations conducted on pentadiene-1,4 by Sverdlov et al. (1958) and on observations made of the IR and Raman spectra of simple unconjugated di-unsaturated molecules (Sheppard & Simpson, 1952).

Semiempirical molecular orbital (SMO) calculations on the simple mono- and poly-unsaturated alkenes listed in Table 2.4.3.3 were performed, and showed similar trends for the C=C stretching vibrations to that observed in the cis unsaturated FAME's. The number of C=C stretching vibrations predicted is equal to the number of isolated double bonds. The higher frequency values obtained using the SMO calculations, shown in Table 2.4.3.4, may be attributed to the semiempirical nature of the calculations, and also the fact that the calculation does not take into account any interactions between adjacent molecules which would suppress the vibrations (i.e. the molecule is effectively isolated). Calculations on simple molecules containing isolated trans double bonds gave results with identical trends to those observed for the cis molecules although the calculated frequencies of the trans bands were lower than those of the cis bands.

Although the frequencies of the C=C stretching bands calculated by the SMO calculations do not agree with experimental results, the predicted number of C=C do agree. Changes in the conformation of a molecule had no effect on the number of C=C stretching bands predicted by the SMO program, however, the relative frequencies were significantly different, as shown by the results for molecule D in Table 2.4.3.4. D\(^{\circ}\), the globular conformation of molecule D, was found to have wavenumber differences between the cis C=C stretching bands which were most similar to those observed for the cis tri-unsaturated FAME's (see Table 2.4.3.1). This suggests that the major conformations of this molecule are globular rather than linear in the liquid or solution state.

The model used to explain the C=C stretching bands observed in the enhanced spectra of cis poly-unsaturated FAME's involved the double bonds lying and vibrating along the same plane with the methylenes attached to the double bonds acting as fixed points. Thus the C-C bonds adjacent to the double bonds offer resistance or enhance the C=C stretching vibrations of adjacent double bonds. This is illustrated by figure 2.4.3.9 which shows the possible modes of vibrations in di- and tri-unsaturated cis compounds. Two interactions are possible between the C=C bonds in a di-unsaturated compound, one in which both the C=C expand and contract at the same time (called sym sym) and the other in which they are out of phase (sym asym).
Table 2.4.3.3: Molecules studied using SMO program

<table>
<thead>
<tr>
<th>SYMBOL USED</th>
<th>STRUCTURE OF MOLECULE</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td></td>
</tr>
<tr>
<td>E</td>
<td></td>
</tr>
</tbody>
</table>
Table 2.4.3.4; Positions of C=C stretching vibrations of molecules shown in Table 2.4.3.3 determined by SMO program.

<table>
<thead>
<tr>
<th>CH=CH</th>
<th>Calculated</th>
<th>difference between bands</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>c</td>
<td>1854</td>
</tr>
<tr>
<td>B</td>
<td>t</td>
<td>1844</td>
</tr>
<tr>
<td>C</td>
<td>c,c</td>
<td>1840</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1849.6</td>
</tr>
<tr>
<td>D^x</td>
<td>c,c,c</td>
<td>1849.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1850.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1859.2</td>
</tr>
<tr>
<td>D^y</td>
<td>c,c,c</td>
<td>1849.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1858</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1861.2</td>
</tr>
<tr>
<td>D^z</td>
<td>c,c,c</td>
<td>1841.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1851.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1860.5</td>
</tr>
<tr>
<td>E</td>
<td>t,t,t</td>
<td>1839</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1842.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1845.6</td>
</tr>
</tbody>
</table>

Where;
c and t are cis and trans double bonds respectively, difference corresponds to wavenumber difference between adjacent bands.

D^x, D^y and D^z represent a linear^a, globular and linear^b conformations of structure D. Where linear^a and linear^b both have linear carbon chains, but the hydrogens are in different conformations.

In the case of the tri-unsaturated compounds three different modes of interactions are possible; sym sym sym, sym sym asym and sym asym sym. In the first case the vibration of the central C=C
bond is restricted by the vibrations of the outer bonds. In contrast, the sym sym asym has two C=C bonds restricting (counteracting) each other and two enhancing each other. Finally, in the sym asym sym mode the central C=C bond vibration is enhanced by the vibrations of the outer C=C bonds. It is difficult to establish which one of these modes of vibration corresponds to which observed band in the IR. In order to assign these modes, normal co-ordinate calculations would have to be performed on the molecules. This facility was unfortunately not available. However, some clues as to the influences of the different C=C bond vibrations were provided by the FAME's studied, in particular the tri- and tetra-unsaturates. For example, when only one methylene is present between the terminal methyl and the double bonds, the position of the highest wavenumber C=C stretching band (which occurs as an inflection) occurs at a lower frequency than in the corresponding FAME's with more than one methylene in this chain. On the basis of the vibrational model proposed above, this observation may be explained by assuming that when only one methylene is present in the terminal methyl chain, the double bond attached to this group has a greater degree of freedom and thus the extent of interaction with the other double bonds is reduced.

The proposed vibrational model is extremely simplistic and does not take into account conformational changes which have been shown to influence the position of the C=C stretching bands in the SMO results. Evidence to support the coexistence of rotational isomers has been provided by Shimanouchi et al. (1971). They studied various model cis and trans compounds containing one or two double bonds in the IR region 600-300 cm⁻¹, and concluded that some of these existed in more than one stable conformation in the liquid or amorphous state. For example cis-3-hexene was found to have two rotational isomers coexisting in the liquid state, one of which is found in the crystalline state. Raman studies of cis-3-hexene in the amorphous solid form (AS) and as crystals (C) by Koyama et al. (1980) revealed the presence of bands corresponding to the C=C stretch at 1657 (AS) and 1661 cm⁻¹ (C). These differences were attributed to differences in the conformation of the molecule. Based on these assignments, they assigned the bands of oleic acid at 1662 (low m.p. crystals) and 1643 cm⁻¹ (high m.p. crystals) to the C=C stretching bands of different stable rotational isomers.

In view of the fact that the C=C stretching bands are of greater intensity in the Raman than the IR, and hence spectral differences more prominent, the literature covering Raman studies of unsaturated fatty acids and their derivatives was re-investigated in order to clarify the observations reported in this section, with respect to the various overlapping cis C=C bands. Only two thorough communications were found. Davies et al. (1972) reported the Raman spectra in CCl₄.
solution of the various positional isomers of octadecanenoic acid and the methyl ester for both the cis and trans isomers. Their results showed that provided that the C=C bond is not conjugated with the carbonyl group, and does not occur on the terminal position, the C=C vibration gives rise to a strong band in the Raman at 1656 ± 1 cm⁻¹ for the cis compounds, and at 1670 ± 1 cm⁻¹ for the trans compounds. No other C=C stretching bands were reported which suggests that in these compounds only one stable rotational isomer occurs in solution or that the frequency differences of the C=C bands arising from different conformations are too small to be resolved. Subsequent Raman studies by Davies et al. (1975) on di-unsaturated acids and methyl esters dissolved in CCl₄, were carried out in order to observe the effect of bringing the two unsaturated groups progressively closer together upon the double bond vibration. They found that provided that there were two or more methylene groups between the double bonds the values were very close to those found for the mono-unsaturated compounds. The cis-9, cis-12 isomer, which has only one methylene between the double bonds, gave a value 5 cm⁻¹ higher than the mono-unsaturated value (1655 cm⁻¹). A similar trend was noted in the present work on the enhanced spectra (see Table 2.4.3.1). This suggests that the cis C=C bands of the cis-9, cis-12 isomer were not resolved in the Raman spectra. Davies et al. (1975) also noted that the position of the C=C band in corresponding trans isomer (trans-9, trans-12) did not differ from that of the mono-unsaturated compounds.

The general conclusion from the literature is that, in solution, the C=C stretching bands corresponding to different rotational isomers have very similar wavenumber values and hence are difficult to resolve. The vibrational model presented above still holds, because the differences in wavenumber attributed to the different vibrational interactions would be greater. However, further investigations are required to confirm this.

According to symmetry considerations, the trans C=C stretching band should be inactive in the IR but active in the Raman. This band has been reported in various Raman studies to occur at 1670 cm⁻¹ (Davies et al., 1972; Davies et al., 1975; Lippert et al., 1972) but has never been observed in the IR. The present IR studies conducted on various mono-unsaturated trans FAME's in solution revealed the presence of a very weak band at 1670 cm⁻¹ which can be attributed to the trans C=C stretching band. The position of this band relative to that of the cis C=C band is shown in figure 2.4.3.10, in which the two bands are overlapped and the carbonyl band subtracted. Although the figure suggests that both the cis and trans bands are of equal intensity, the actual intensity of the cis band is much greater than that of the trans band. The appearance of the trans band in the IR is probably due to the difference between the methyl ester chain and the methyl chain on either
side of the double bond, which reduces the symmetry.

Examination of the spectrum (figure 2.4.3.11) of the 7th fraction from the alkaline-isomerisation experiment (section 2.3.10), showed the presence of 6 weakly absorbing bands in the region 1680 to 1550 cm\(^{-1}\). The positions of these bands are shown in Table 2.4.3.4. This fraction consists of at least 5 components (conjugated/unconjugated isomers of methyl linoleate) which are present in varying amounts (see Table 2.3.10). Four of these isomers present are unidentified. These bands can be ascribed to the C=C stretching vibrations of the various isomers present, by analogy with those observed for the isomers of hex-2,4-dienoic acid and methyl deca-2,4-dienoate (see Table 2.2.4.2). The intensity of the C=C stretching bands of the hex-2,4-dienoic acid and methyl deca-2,4-dienoate isomers is enhanced due to conjugation with the carbonyl bond, which probably also shifts the frequencies of these bands and explains why they do not correspond exactly with those reported in Table 2.4.3.4.

Table 2.4.3.4; Positions of C=C stretching bands in spectrum of 7th fraction from alkaline-isomerisation of methyl linoleate. s = inflection.

<table>
<thead>
<tr>
<th>C=C band</th>
<th>wavenumber (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1652</td>
</tr>
<tr>
<td>b</td>
<td>1650-1640s</td>
</tr>
<tr>
<td>c</td>
<td>1626</td>
</tr>
<tr>
<td>d</td>
<td>1612</td>
</tr>
<tr>
<td>e</td>
<td>1601</td>
</tr>
<tr>
<td>f</td>
<td>1581</td>
</tr>
</tbody>
</table>

2.4.4 Region 3 - 1600 to 1000 cm\(^{-1}\)

This region contains a wealth of spectral information. Unfortunately the extent of overlap between bands is too great, except in the case of the solid phase spectra (progression bands). Even in the solid phase spectra the problem of band overlap prevents any useful information being obtained
from mixtures containing more than two components. With this in mind, the deconvoluted spectra of various FAME's in the liquid phase and in solution were studied in order to establish whether any useful bands, not apparent in the "normal" spectra, are enhanced by this technique. These studies showed that in the "normal" spectra of cis FAME's with isolated double bonds, the position of the band attributed to the in-plane =C-H deformation shifts to lower frequency with increase in unsaturation (see Table 2.4.4.1). This has not been reported previously in the literature, only the fact that this band increases in intensity with unsaturation. Deconvolution improved the resolution between the bands in this region and disclosed that the cis mono-unsaturated FAME's have one in-plane =C-H deformation band, while the same band in the di-FAME's is unsymmetrical and of greater intensity. This is relative to the band at 1419 cm\(^{-1}\) which corresponds to the methylene adjacent to the carbonyl group which should not change in intensity between different categories of FAME's. The asymmetry of the in-plane =C-H deformation band of the di-unsaturated FAME's was considered to be due to the presence of two closely overlapping bands. These arise because the =C-H groups are in two different environments. For example, there are two equivalent =C-H groups adjacent to the methylene chains (referred to as type A) and another two equivalent =C-H groups adjacent to the methylene groups separating the double bonds (referred to as type B).

Table 2.4.4.1; shows the positions of the cis in-plane =C-H deformation bands of various categories of FAME's observed in the normal and deconvoluted spectra.

<table>
<thead>
<tr>
<th>type of cis FAME</th>
<th>position(s) of cis in-plane =C-H deformation bands (cm(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>normal spectra</td>
</tr>
<tr>
<td>mono-</td>
<td>1404</td>
</tr>
<tr>
<td>di-</td>
<td>1398</td>
</tr>
<tr>
<td>tri-</td>
<td>1394</td>
</tr>
<tr>
<td>tetra-</td>
<td>1393</td>
</tr>
</tbody>
</table>

A further explanation being considered for the asymmetry of the =C-H deformation band was that it was an artefact of the deconvolution technique, however this was quickly disproved by the deconvoluted spectra of the cis isolated tri-unsaturated FAME's which all showed the presence of
two partially resolved in-plane =C-H deformation bands of similar intensity. The lower frequency band is assumed to correspond to the in-plane =C-H deformation of type B groups, because the intensity of this band increases with increase in the degree of unsaturation, and hence the number of =C-H groups of this type. Evidence to support this assumption was provided by the deconvoluted spectrum of 22:4Δ7c10c13c16c, containing four isolated double bonds, which showed one unsymmetrical band with a maximum corresponding to the lower frequency in-plane =C-H deformation band (type B). This is, the expected result of the higher proportion of type B =C-H groups present. Figures 2.4.4.1 to 2.4.4.4 show the enhanced spectra of 18:1Δ9c, 18:2Δ9c12c, 18:3Δ9c12c15c and 22:4Δ7c10c13c16c which represent mono-, di-, tri- and tetra-unsaturated cis FAME’s, respectively. The spectra substantiate the observations referred to above.

Trans FAME’s do not possess a band in the region of the cis in-plane =C-H deformation, as demonstrated by the enhanced spectrum of methyl elaidate (see figure 2.4.4.5). Raman studies by various authors (see section 2.2.2) have shown that the corresponding trans in-plane =C-H deformation occurs between 1310 - 1250 cm⁻¹. Comparison of corresponding sets of cis/trans isomers showed that differences were apparent in this region. However, because of the extent of overlap between bands it was impossible to assign bands to any particular vibrational mode (see figures 2.4.4.1 and 2.4.4.5).

Some similarities were observed between the progression bands of the enhanced liquid spectra of cis and trans mono-unsaturated FAME’s, containing an equal number of methylenes in the chain between the carbonyl group and the double bond. The number of methylenes in the chain between the terminal methyl group and the double bond appears to have little or no effect upon this region. This is as predicted for the solid phase spectra (see section 2.2), the deconvolution technique facilitating the observation of these bands. Poly-unsaturated FAME’s do not have progression bands resembling those of the mono-unsaturated FAME’s with an equivalent chain segment attached to the carbonyl group. Further investigations into this area were discontinued because it was considered impossible to extract any meaningful information from spectra of FAME mixtures. Spectral differences were observed in the region 1500 to 1400 cm⁻¹, where the bending vibration of a methylene group adjacent to a double bond is assumed to absorb, but once again further investigations were not warranted on account of the degree of overlap between bands.

In the region 1150 to 1050 cm⁻¹ spectral similarities were found between corresponding cis/trans isomers of mono-unsaturated FAME’s (e.g. 18:1Δ9c and 18:1Δ9t). The bands that occur in this
region have been assigned to the skeletal vibrations of the polymethylene chains on either side of the double bond, coupled with other vibrations. Furthermore, some bands in this region were common to both cis and trans isomers having identical numbers of methylenes in the chain attached to the carboxyl group. This is illustrated by figure 2.4.4.6 which shows the spectra of various mono-unsaturated FAME's in this region. Koyama and Ikeda (1980) studied the skeletal vibration bands of the polymethylene chains of cis- and trans-unsaturated fatty acids in the Raman spectrum between 1130 - 970 cm⁻¹ (solid phase). They observed, that for an acid \( \text{CH}_3(\text{CH}_2)_M-2\text{CH} = \text{CH}(\text{CH}_2)_N-2\text{COOH} \), the frequencies of skeletal bands for those acids having the same number of methylenes in the carboxyl chain (N-2), but different numbers of methylenes in the methyl chain, were similar to each other. This is in agreement with the observations made in the present work (previous paragraph). However, skeletal bands corresponding to the methyl-chain were also observed, by Koyama and Ikeda, but were not observed in the present work (probably due to the difference in state, i.e. in the liquid phase the bands broaden and thus there is a greater degree of overlap). Koyama and Ikeda concluded that the skeletal stretching vibrations in this region are localised within each polymethylene chain, and they explained these bands in terms of the set of phase differences \( k = km/M \) and \( k = kN \) \((k = 1, 2, \ldots; M \text{ and } N \text{ correspond to } \text{CH}_3(\text{CH}_2)_M-2\text{CH} = \text{CH}(\text{CH}_2)_N-2\text{COOH})\). Hence, these bands can be used to establish the length of the polymethylene chains on either side of the double bond from an examination of the Raman spectrum of mono-unsaturated fatty acid in the solid phase. Liquid phase IR spectra can be used to establish the approximate number of methylenes in the carboxyl chain only.

2.4.5 Region 4 - 1000 to 400 cm⁻¹

Most of the work on the IR spectra of unsaturated isomers cited in the literature has concentrated on the region 1000 to 400 cm⁻¹, where the distinct trans double bond \( =\text{C-H out-of-plane deformation vibration occurs. This section will not discuss this band, which will be covered in detail in the following chapter, but rather other spectral features which have either not been reported in the literature or have not been fully investigated, because they were considered unsuitable for quantitation.}

The first of these spectral features involves a series of overlapping bands which occur between 910 to 800 cm⁻¹. These have been assigned tentatively to the methyl rock of the methyl ester group \(-\text{COOCH}_3\) by various authors (see section 2.2.1). This was confirmed by the spectrum of
deuterated methyl stearate (CH₃-(CH₂)₁₆-COOCD₃) in which these bands are absent (see figures 2.4.5.1 and 2.4.5.2 representing methyl stearate and the deuterated isomer, respectively). However, the trans mono-unsaturated FAME's which have the same number of methylenes in the polymethylene chain attached to the carboxyl group - 14:1Δ9t, 16:1Δ9t and 18:1Δ9t, all had similar profiles of overlapping bands in this region. This was irrespective of the number of methylenes in the methyl chain, whereas those having different numbers of methylenes in the carboxyl chain possess different profiles. An identical trend was also observed for the cis mono-unsaturated FAME's studied. Figure 2.4.5.3 shows the deconvoluted spectra of various trans FAME's in the region 950 to 750 cm⁻¹ which illustrate the trend. Two possible suppositions which could explain the above observations are: (a) these bands arise from the coupling of the methyl rock (−COOCH₃) and vibrational modes of the polymethylene chain attached to the carboxyl group, and (b) bands resulting from the carboxyl chain methylenes absorb in this region, and overlap with those of the methyl rocking mode.

A general resemblance between the bands in the region 950 to 750 cm⁻¹ was also observed between corresponding cis/trans isomers (e.g. 18:1Δ9c and 18:1Δ9t), the only exceptions being 18:1Δ6c and 18:1Δ6t. Comparison of the deconvoluted spectra confirmed these bands similarities between cis/trans isomers, as illustrated by figure 2.4.5.4, which shows the deconvoluted spectra of 18:1Δ9c and 18:1Δ9t. In addition, these spectra revealed that all the cis isomers had an overlapping band at 855 cm⁻¹ which was not present in trans isomers. This band has not been cited in the literature and is presumably associated with the cis double bond.

Poly-unsaturated FAME's liquid phase spectra in the region 950 to 750 cm⁻¹ were significantly different from those of the mono-unsaturated FAME's. No correlation was established between the bands in this region and the structures of the poly-unsaturated FAME's studied, apart from the band at 913 cm⁻¹. This was found to be present in the spectra of all the cis di- and tri-unsaturated FAME's studied, and not in the effect of the corresponding trans isomers (see figure 2.4.5.5 - deconvoluted spectra of 18:2Δ9c12c and 18:3Δ6c9c12c). In the case of the cis tri-unsaturated FAME's this band overlaps with smaller bands not present in the di-unsaturated FAME's and hence are not that well resolved. Jackson et al. (1952) were the first to report the presence of a band at 913 cm⁻¹ in the IR spectrum of methyl linoleate in their spectral study of this compound, and its various conjugated/unconjugated isomers. This band was absent in the spectra of conjugated/unconjugated isomers, in agreement with the results obtained from the isomerisation experiment where this band was found to decrease with increase in isomerisation (see figure 2.4.5.6). Figure 2.4.5.7 represents the spectra of methyl linoleate (starting material) and the 4th
and 7th distillation fractions, from the alkaline isomerisation experiment (section 2.3.10), in the region 1050 to 400 cm\(^{-1}\). The figure clearly shows that bands A (983 cm\(^{-1}\)) and B (946 cm\(^{-1}\)), which are assigned to the \(\equiv\text{C-H}\) deformation of the conjugated cis/trans isomers of methyl linoleate, increase in intensity with increase in boiling point of the fraction. This would be predicted, since the conjugated isomers have the highest boiling points and would therefore be more concentrated in the higher fractions. Other evidence which confirms that the methyl linoleate has been isomerised is the narrowing and decrease in intensity of the band at 720 cm\(^{-1}\) and appearance of a new band at 810 cm\(^{-1}\). The band at 720 cm\(^{-1}\) is assigned to the methylene wagging mode, however it is known to be broader and of greater intensity in cis FAME's, due to the overlap of a band tentatively assigned to the cis \(\equiv\text{C-H}\) out-of-plane deformation. This assignment is supported by the observations mentioned above, in which an increase in the cis/trans conjugated isomers (or loss of cis bonds) leads to a lowering in intensity and narrowing. The appearance of the new band at 810 cm\(^{-1}\) is of particular interest since it is not cited in any of the literature. The assignment of this band is uncertain.

Attempts at resolving the cis \(\equiv\text{C-H}\) out-of-plane deformation from the methylene wagging band in the spectra of cis FAME's by using the deconvolution technique were unsuccessful. Figures 2.4.5.8 and 2.4.5.9 show the normal and deconvoluted spectra of methyl oleate and methyl linoleate, illustrating the extent of overlap between these bands. Finally, tri-unsaturated cis FAME's were all found to possess a band at 793 cm\(^{-1}\), which was not found to be present in any other category of FAME's. No reference to this band was found in the literature.

### 2.5 SUMMARY/CONCLUSION

The foregoing discussions show that the cis-isomers studied can be readily distinguished from the trans-isomers by their characteristic IR absorption spectra, in particular by the presence or absence of the prominent cis \(\equiv\text{C-H}\) stretching band (3005 to 3010 cm\(^{-1}\)) and the trans out-of-plane \(\equiv\text{C-H}\) deformation band at (969 cm\(^{-1}\)). These bands are well documented, and form the basis for the quantitative IR determination of the cis/trans content of fats and oils. Unfortunately, cis/trans poly-unsaturated FAME's possess these bands too, introducing an error into the calculated values. The errors present in both the quantitative cis and trans IR methods described above are discussed in more detail in the Chapter 3.
As part of the present work, studies were conducted to establish alternative bands present in the IR spectrum which are associated with particular cis/trans unsaturated FAME’s, and which can be measured quantitatively using the superior FTIR technique to overcome the problems associated with the current IR methods. In addition, the spectral study addresses the problem of identification of similar unsaturated FAME’s. Although a number of bands were assigned to the trans double bond which were previously unreported in the literature, no alternative bands were found in the IR spectrum that could be used to distinguish between the trans FAME’s containing different numbers of isolated double bonds. In the case of the cis FAME’s studied, a greater number of previously unobserved bands and spectral features associated with the isolated cis double bond(s) were discovered. The larger number of cis bands relative to trans bands present in the IR spectrum is due to symmetry considerations. Spectral dissimilarities between the cis FAME’s containing different degrees of unsaturation were observed for the C=C stretching vibration and the =C-H in-plane deformation. These differences were enhanced in the deconvoluted spectra. The frequency of the C=C stretching vibrations also provides some information about the location of the double bonds in poly-unsaturated FAME’s. Two other bands associated with cis poly-unsaturated FAME’s are the bands located at 913 cm⁻¹ and 793 cm⁻¹. The former band is present in both the di- and tri-unsaturated cis FAME’s studied, while the latter only in tri-unsaturated cis FAME’s. Both these bands remain unassigned.

Some of the bands described above will be assessed, in the following chapter, to establish whether they can be used quantitatively to determine the mono-, di- and other poly-unsaturated cis FAME’s content of natural and synthetic mixtures of saturated and cis/trans FAME’s. The results will be compared with those of the current cis/trans IR methods as well as a capillary GC method, in order to establish which gives the most reliable results. The following criteria were considered in the assessment of the various methods (which will be discussed in detail in Chapter 3):

- Specificity
- Sensitivity
- Accuracy
- Precision
- Robustness

One of the major problems with the spectra of cis and trans FAME’s in either solution or the liquid state, is that they closely resemble the spectra of other corresponding cis and trans positional or
different chain length isomers. This problem tends to be more acute for mono-unsaturated FAME's than poly-unsaturated FAME's which tend to have distinctive features that can be used to identify them. Preliminary studies have shown that three spectral regions (see Table 2.5.1) could be used to provide information regarding the position of the double bond in the methylene chain of cis or trans mono-unsaturated FAME's. All of these regions require spectral deconvolution to enhance distinguishing features, and unfortunately only provide information about the number of methylenes in the chain attached to the carboxyl group. This line of investigation was not pursued due to the fact that these regions would provide no useful information about individual FAME's in mixtures because of the difficulty of resolving several sets of overlapping bands.

Table 2.5.1; Spectral regions in the deconvoluted spectra of mono-unsaturated FAME's which can be used to establish the number of methylenes in the chain between the carboxy group and the double bond.

<table>
<thead>
<tr>
<th>Region</th>
<th>Spectral range of bands</th>
<th>Assignments</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1500 -1150 cm⁻¹</td>
<td>Progression bands</td>
</tr>
<tr>
<td>2</td>
<td>1130 - 970 cm⁻¹</td>
<td>Skeletal vibrations of the polymethylene chains</td>
</tr>
<tr>
<td>3</td>
<td>910 - 800 cm⁻¹</td>
<td>Methyl rock of the -COOCH₃ group</td>
</tr>
</tbody>
</table>
CHAPTER THREE

FTIR Quantitative Studies of Cis/Trans FAME's

3.1 INTRODUCTION

Investigations described in Chapter 2 have shown how the FTIR spectrometer's superior signal-to-noise ratio, coupled to data processing capabilities, enabled the observation of previously undetected bands, and the enhancement of spectral features which were not apparent using conventional dispersive instruments. This chapter describes how some of these bands and spectral features were used to establish the cis/trans content of fatty acids. A comparison is made with the currently accepted IR and GC methods.

Before attempting to assess the FTIR techniques, the author initially reviewed the current GC and IR techniques for quantitative analysis of trans content of fats and oils (see section 3.2). The difficulty in comparing the advantages and disadvantages of two totally different techniques soon became apparent. This was exemplified by the comparative studies between IR and GC techniques reported in the literature, which concentrated on one aspect, namely, how well the results obtained by each method fitted the known values. The samples used in these studies sometimes varied in composition - for example trans content, interfering fatty acids and on occasions the distribution range of different chain length fatty acids. These variables were introduced in order to establish the influence of various factors upon the performance of each technique. However, the variables were not always well controlled and thus resulted in little or no useful information being obtained about these factors. An alternative approach was called for, which could categorise the advantages and disadvantages of the two totally different techniques in a manner conducive to comparison. One systematic evaluation scheme used extensively by chromatographic chemists was considered appropriate for this task. According to this scheme, the validation of quantitative methods has to address the following; specificity, sensitivity, accuracy, precision and robustness (ruggedness).

Specificity is required to demonstrate that the analyte (trans fatty acids) is detected and quantified in a sufficiently specific manner, free from interferences from other fatty acids or other
Sensitivity is related to a method's ability to respond to small changes in the amount of analyte present. The qualitative limit of detection (usually three times the level of the background noise) as well as the minimum quantifiable level, are normally taken as a measure of sensitivity.

Accuracy is the closeness of experimental results to the true value; precision is the closeness of results to each other following replicate analyses. Precision concerns both repeatability and reproducibility. The first involves multiple analyses of the same preparation by the same operator using the same apparatus in the same laboratory over a short period; the second involves multiple analyses of different samples by different operators in different laboratories over a longer period.

Robustness is the method's ability to withstand minor changes in experimental or environmental conditions without affecting reliability. This is sometimes achieved at the expense of simplicity of use. Simplicity of use is an important factor because it determines how well a method will be accepted by the end users.

One vital factor must be taken into consideration, and that is, what the method has to achieve. The objective of the method is that it must be able to determine the trans fatty acid content of food fats. However, since the trans and saturated fatty acids are effectively classified as the same, an alternative approach to this problem can be used, namely, a method which determines the cis mono- and poly-unsaturated fatty acid content. It is then a simple matter of subtracting the cis mono- and poly-unsaturated fatty acid content from the total fatty acid content to establish the combined trans and saturated fatty acid content. Bearing this in mind, new FTIR methods used to determine the cis content were also assessed.
3.2 LITERATURE REVIEW OF THE DETERMINATION OF CIS/TRANS FATTY ACIDS

3.2.1 Determination of trans fatty acids by infrared spectroscopy

All of the IR methods used to establish the trans content are based on the quantitative measurement of the absorption band at approximately 970 cm⁻¹. Various authors have assigned this band to the out-of-plane =C-H deformation about a trans ethylenic double bond (see section 2.2.3).

Shreves et al. (1950a and 1950b) were the first to employ this band for the determination of the isolated trans bond content in saturated and mono-unsaturated octadecenoic acids and related compounds. Extinction coefficients (k) were reported for saturated and for cis and trans mono-unsaturated fatty acids, methyl esters, triglycerides and alcohols dissolved in CS₂ and used to calculate the trans composition of mixtures. Shifts in the double bond from Δ⁹ to Δ⁶ positions in either the cis or trans octadecenoic acids were found to produce a small change in the extinction coefficient. Sheres et al. assumed that an equivalent shift in the opposite direction to Δ¹² or any intermediate position would likewise produce little change. Recommendations were made that the effects of more drastic shifts along the chain on k be investigated.

The method of calculating the trans content developed by Shreves et al. incorporated a correction factor in order to compensate for the small absorption in the trans region by both the saturated and cis mono-unsaturated compounds in mixtures. The formula worked out by Shreves et al. for a mixture of cis and trans unsaturated components is shown in equation 3.2.1.

\[
\text{Eqn. 3.2.1} \\
\text{Trans components weight %} = \frac{100(K_{ob} - K_{cis})}{K_{t} - K_{cis}}
\]

\[K_{ob} = \text{observed extinction coefficient of the fatty acid mixture}\]
\[K_{t} = \text{extinction coefficient of the trans unsaturated fatty acid}\]
\[K_{cis} = \text{extinction coefficient of the cis unsaturated fatty acid}\]

Results on unknowns of methyl esters, glycerides or alcohol mixtures may be similarly calculated by using appropriate k values for that class of compound. The corresponding formula for
mixtures of trans unsaturated substances and saturated substances has $K_{cis}$ replaced by $K_{sat}$.

Equation 3.2.2 was used to deal with mixtures containing cis unsaturated and saturated as well as trans unsaturated substances.

Eqn. 3.2.2

$$\% \text{ trans} = \frac{100(k_{ob} - k_{cis}y - k_{sat}z)}{(kT - k_{cis})}$$

$y$ = total weight of the C18 unsaturated compound fraction
$x$ = weight of the saturated components
$y$ is calculated from the iodine number of the mixtures and $z$ by difference.

This latter equation was further simplified by using the mean values since the coefficients of the saturated and cis-unsaturated components are very similar (see equation 3.2.3). Results obtained using this equation were accurate on octadecenoic acids in the $\Delta^6$ to $\Delta^{12}$ range.

Eqn. 3.2.3

$$\text{Trans components weight} \% = \frac{100(k_{ob} - k_{mean})}{(k_T - k_{mean})}$$

$k_T$ = appropriate average $k$ value of two trans fatty acids
$k_{mean}$ = the average $k$ value of various saturated and cis mono-unsaturated fatty acids
$k_{ob}$ = the observed $k$ value of the sample

Results on unknown of methyl esters, glycerides or alcohol mixtures may be similarly calculated by using appropriate $k$ values for that class of compound.

An identical mathematical treatment of the IR data was proposed by Ashler et al. (1953). In this study, the trans content was determined, and the method extended to cope with conjugated unsaturation by the introducing of an additional correction factor calculated from UV data. The study was based on solution phase IR spectra of some 19 fatty acids, including mono-unsaturated acids as well as poly-unsaturated acids, conjugated and non-conjugated (all these fatty acids contained 18 carbons in the fatty acid chain). This work confirmed the additive effect of the trans absorption bands in poly-unsaturated fatty acids containing non-conjugated as well
as conjugated trans double bonds. Furthermore, a shift in the frequency of the trans band to longer frequencies was observed to occur progressively for trans-conjugated systems with increase in conjugation. Conjugated compounds containing both cis and trans bonds were observed to contain two bands in the trans region similar to those reported in section 2.2.4, however, the samples concerned were known to be mixtures, so little emphasis was placed on the assignment of these new bands. No allowances were made for the relative purities of the samples used in this study. This fact was used to explain for the apparent poor agreement of the extinction coefficients of trans band at 970 cm⁻¹ between the mono-, di- and tri-unsaturated trans fatty acids (i.e. these values were not exactly multiples of the mono-unsaturated trans).

Jackson et al. (1951) used an alternative "baseline" technique for the determination of sample and pure trans compound absorption at 965 cm⁻¹. This technique does away with a solvent absorption measurement for each determination, and corrections for cis unsaturated and saturated compounds are not necessary. In practice, the sample is weighed into a volumetric flask made up to volume with carbon disulphide and the infrared spectrum recorded over the 1050 to 900 cm⁻¹ region. A baseline is drawn (see figure 3.2.1.1) and the absorbance due to trans double bonds then compared with the absorbance of a known standard sample. The trans double bonds content is calculated as a percentage weight of that standard sample (i.e. methyl elaidate, elaidic acid or trielaidin).

A report by the Spectroscopy Committee of the American Oil Chemist's Society (1957) presented results of a collaborative study of the determination of trans unsaturation by the "baseline" method, and that proposed by Shreves et al. (1950a). Collaborators analysed six fat samples and the methyl esters prepared from these samples. The report stated that good results could be expected if a specific set of experimental procedures were followed. Furthermore, if acids are to be analysed, the standard should be elaidic acid; if esters, methyl elaidate; and if triglycerides, trielaidin should be used. Lower results were noted after conversion to the methyl esters using both methods. The Committee did not investigate the cause of the differences, and said only that samples should not be converted into another form (e.g. into fatty-acid methyl esters) and did not recommend either of the methods.

Kaufmann et al. (1959) subsequently demonstrated that the decrease in trans content obtained after conversion of triglycerides to methyl esters could be accounted for by the absorption of the glyceride structure at ≈ 970 cm⁻¹. The acids and methyl esters also absorb in the same region, but the latter to a lesser degree. Kaufmann et al. recommended that methyl esters be used in preference to either the glycerides or acids in the infrared determination of trans content.
These conclusions were confirmed by studies conducted by Firestone et al. (1961, 1965) and in the 1958-1959 report of the Spectroscopy Committee of the American Oil Chemist's Society (1959). In the latter work, results of a collaborative study of a proposed "baseline correction" infrared method for trans determination were reported. A series of triglycerides, methyl esters, and acid samples were analysed by twelve collaborators who were also supplied with elaidic acid, methyl elaidate and trielaidin standards. Results on the analyses of the acids did not show satisfactory agreement and the recommended method did not include the analysis of acids. Recommendations were made that the fats be converted to the methyl esters and then analysed to eliminate the bias introduced by the glycerides. The procedure used in the "baseline" technique was used as the basis of the Tentative Method Cd 14-61 by the American Oil Chemist Society (1978).

Firestone et al. (1961, 1965) studied the "baseline" technique, using samples or standards in the form of either methyl esters or glycerides. They established that triglycerides with less than 10% of trans components produced trans content values which were about 2-3% higher, whereas methyl esters derivatives produced values which were about 1.5-3% lower than the known values.

An alternative "differential" technique has been described by various authors (Hartman et al., 1958; Hartman et al., 1955; McDonald, 1954; Cleverley, 1960; Huang et al., 1971a). Compensation for the interfering absorbances of acids and methyl esters in the trans region were achieved by the use of a sample cell containing the acid or methyl ester sample in CS$_2$ and an additional reference cell with an equal thickness and concentration of a corresponding reference compound (normally stearic acid or methyl stearate), which are placed in the sample and reference beam of the IR spectrometer and the spectrum taken. Adjustments were necessary to counteract over or under compensation which occur due to differences in cell pair transmittance and thickness. The background from which the extinction was measured was obtained by recording the spectrum of the sample cell containing carbon disulphide, with the references cell containing the same thickness of solvent.

Huang et al. (1971a) compared the results obtained using the "differential" procedure with that of the "baseline" method. The samples used contained low levels of isolated trans isomers (1-12%) in vegetable oils (triglycerides) or derived methyl esters. The "differential" procedure was found to be more accurate than the "baseline" method, which is known to produce a positive bias for the triglycerides (2-3% higher) and a negative bias for the derived methyl esters (1.1-
1.6% low). Studies were also conducted on the "differential" procedure in which the trans content of an oil, spiked with trans isomer, was determined using an equivalent concentration of a different oil in the reference cell. The results suggested that if an equal weight of a different oil, or the derived methyl ester, is used in the reference cell, the results obtained by the method are comparable to those obtained when the sample oil/methyl ester is used as a reference. Although several oils were used in this study, the predominant fatty acids in all of them contained 18 carbons in the chain. The present author questions the suitability of this procedure for fats and oils whose composition is unknown and does not consist predominantly of fatty acids with eighteen carbons in the acid chain.

A rapid method for the determination of trans unsaturation in fats was developed by Allen (1969) from the measurement of the infrared bands at 1169 cm⁻¹ and 965 cm⁻¹, corresponding to the ester group (see section 2.2.1) and the trans double bond, respectively. The basis of this method is that the ratio of the absorbances of the two bands has a linear relationship with the number of trans double bonds. An identical relationship was also shown to occur for acids and triglycerides, using the respective reference bands (acid - 930 cm⁻¹; triglyceride - 1153 cm⁻¹). Only a small quantity of sample need be dissolved in the solvent (carbon disulphide was recommended) and the IR spectrum recorded using a reference cell filled with the solvent. To calculate the percentage trans content in the sample, the ratio of the two absorbance values was substituted into a linear equation (see equation 3.2.4) which was developed from known standard mixtures (methyl oleate + methyl elaidate). The method's main advantage was that of speed, because the sample need not be weighed nor made up to a known volume. Comparison of the linear equations developed from methyl esters of different carbon chain lengths in the fatty acid fragment, revealed that the absorptivity at 1169 cm⁻¹ increased as the molecular weight of the ester decreased. Thus, the equation must be developed from known samples that are similar to the unknown.

\[ \text{Eqn. 3.2.4} \]

\[
\% \text{ Trans} = K(A_{965}/A_{1169}) - f
\]

Where \( K \) and \( f \) are the gradient and intercept of the standard calibration plot, \( A \) is the absorbance.

A comparative study by Huang et al (1971b) was made between the rapid method reported by Allen (1969) and the tentative method of the American Oil Chemist's Society for the
determination of isolated trans isomers ("baseline" method, 1970). The rapid method was found to be less accurate than the "baseline" method. However, the accuracy of the rapid method was shown to be improved when an oil or methyl ester matrix with the same composition as the sample matrix was used to establish the calibration plot.

Ideally, analysis of a sample as a triglyceride and as a methyl ester should give identical trans values. Firestone et al. (1965) addressed this situation by proposing a correction for the positive triglyceride absorption and negative methyl ester absorption encountered with samples containing low trans content (<15%) using the "baseline" correction technique. Another solution proposed by Madison et al. (1982) involved the use of the "baseline" correction technique in conjunction with a two-component calibration standard mixture to produce the calibration plot and measuring the samples as methyl esters (i.e. methyl elaidate and methyl linoleate mixtures). Trans content results obtained using this procedure were compared against those obtained by two other IR techniques, the "baseline" correction technique and the AOAC method (1975), and by a capillary GC method. The procedure was shown to be more accurate than the other two infrared techniques, eliminating the need for calculation factors in the AOAC method and correcting for the high bias of the "baseline" correction technique. Good agreement between the observed trans content values obtained by the proposed IR method and the GC technique was demonstrated. The comparability of the trans values determined by this method with the analysis as triglycerides and as methyl esters was demonstrated. The procedure used on the triglycerides was identical to that of the methyl esters, differing only in that tristearin was substituted for methyl linoleate and trielaidin for methyl elaidate in the preparation of the standard solutions. The trans content values obtained using this procedure on either triglycerides or methyl esters were almost identical, which cannot be said for any of the other IR techniques. The two-component standard mixture using methyl esters was chosen as the preferred method of measurement, because the methyl ester value was considered more accurate.

Three papers describing the determination of the trans content using FTIR techniques have been reported. The first, by Sleeter (1987), reported the use of FTIR to increase the accuracy of the measured trans content by the "baseline" correction technique. This was achieved by using the area of the trans peak instead of its height, and a second order polynomial fit for the calibration plot. The second of these papers, by Lanser et al. (1988), was also based on peak areas. Integration of peak area was performed using various baselines. They established that different baseline parameters were required, according to the width of the band, thus
compensating for the band broadening observed at higher trans content. Studies were conducted on various standard mixtures and hydrogenated vegetable oils. The results for percentage trans were compared with the corresponding results obtained by capillary gas chromatography, and by the IR peak height "baseline" correction technique. Lanser et al. concluded that the accuracy of the FTIR area method was comparable to the capillary GC method, and better than the IR "baseline" method. Finally, Sleeter et al. (1989) proposed an FTIR method which analysed neat methyl ester samples, eliminating the need for dilution by a volatile solvent. A method involving peak areas was used, in which the baseline parameters were varied automatically by a computer program. They showed that the accuracy of this procedure was better than that of the peak height "baseline" correction technique using a dispersive instrument. Only standard mixtures were studied; the method was not used on "real" samples.

Two methods using Attenuated Total Reflectance (ATR) to establish the presence of the isolated trans double bonds in oils and fats have been described (Dutton, 1974; Belton et al., 1988). The advantages of these methods over methods using transmittance spectra centre on the fact that the samples do not need to be dissolved in a solvent or derivatized to the methyl esters. Furthermore, the presence of water in samples (e.g. margarines) was found to have no effect upon the ATR spectra. In contrast, the presence of water gives rise to severe problems in transmission work, and samples must be dried before analysis. However, it is well known that the Beer-Lambert law is not obeyed by ATR results, except over narrow concentration ranges (Wilks, 1972). This was overcome by fitting a polynomial to the calibration plot. Another problem encountered involved the poor repeatability between replicate samples as a result of inconsistent coverage of the ATR crystal. In order to overcome this problem Belton et al. (1988) proposed the use of an internal peak ratioing method, similar to that described by Allen (1969), in which the absorbance of the trans band was ratioed against that of the carbonyl stretch at 1743 cm\(^{-1}\) (methyl ester). In this way the random variations in absorbance were eliminated.

Only one method of determining the trans content in the solid phase has been described in the literature (Kaufmann et al., 1959). This method involves the IR measurement of Ba salts of the fatty acids in KBr discs. Spectra obtained by this technique showed none of the interfering bands observed in the trans band region present in triglycerides, acids or methyl esters. However, in order to obtain an accurate results using a dispersive instrument, Kaufmann et al., showed that the trans band must be in the 20-60% transmission range. Samples possessing low trans levels require large quantities of sample to be incorporated into the KBr disc in order to meet this criterion resulting in opaque discs and a high level of scattering. Another factor which
has reduced the popularity of this method is the inconvenience of KBr disc preparation.

The above paragraphs are re-evaluated below in terms of the five criteria put forward in the introduction to this chapter.

Specificity; All compounds containing double bonds in the trans configuration possess a band at approximately 967 cm$^{-1}$. This includes non-conjugated and conjugated poly-unsaturated compounds, which means that this band provides little or no information about the position of the double bond, the fatty acid chain-length distribution or proportions of mono- and poly-unsaturated trans components. Furthermore, other bands present in saturated and cis-unsaturated FAME's also absorb in this region (see section 2.2.1). The absorbance of these bands is weak in comparison to that of the trans band, but the bands do cause problems in the determination of the trans content of fats and oils containing low levels of trans components. Most of the publications have recognised this important issue and attempt to resolve it using various correction techniques.

Sensitivity; This criterion is dependent upon the composition of fat or oil and the ability of the IR technique to compensate for the absorbance of the interfering bands. Limits of quantitation as low as 1% (w/w) have been claimed.

Accuracy; Good results have been obtained using fats and oils containing methyl elaidate and methyl oleate as the predominant unsaturated FAME's. In the case of hydrogenated samples (which contain trans isomers besides methyl elaidate: e.g. positional, different fatty chain-lengths and trans isomers varying in the degree of unsaturation) studies of accuracy have not been conducted, so the effect of determining the trans contents as the percentage weight of methyl elaidate has not been established (i.e. whether the extinction coefficient of the trans band of the trans isomers present are the same as that of methyl elaidate, and if not what effect does this have). This is due to the difficulty of establishing the true composition of these samples.

Precision; Good results have been obtained for both repeatability and reproducibility using various IR techniques.

Robustness; The IR techniques appear to be easily transferable to other laboratories.
3.2.2 Determination of cis FAME's by infrared spectroscopy

Sinclair et al. (1952b) demonstrated that the band at 3020 cm\(^{-1}\), assigned to the cis C-H stretch, increased with the number of cis double bonds present, while the methylene peak at 2920 cm\(^{-1}\) diminished. They suggested that these observations could be used as the basis for the evaluation of cis FAME's using a calibration plot of the absorbance ratio \((2920 \text{ cm}^{-1}/(2920 \text{ cm}^{-1} - 3020 \text{ cm}^{-1}))\) vs. cis content, established from calibration mixtures of saturated and cis-unsaturated FAME's. This suggestion was reiterated by Chapman (1965).

Arnold et al. (1971) showed that a linear relationship exists between the absorbance ratio (absorbance of the 3020 cm\(^{-1}\) band divided by the absorbance of another characteristic triglyceride band) and the iodine value determined for various fats and oils. Estimations of the iodine value of 19 additional fats and oils by the IR technique (using the calibration plot of the other samples) and the standard Wijs method revealed good agreement. Anderson et al. (1974) also demonstrated that a linear relationship exists between the absorbance of the band at 3020 cm\(^{-1}\) and the iodine value of various vegetable oils and butter oils. The absorbance of the band was determined using the differential technique in which a reference cell containing a standard (tristearin) at a similar concentration as the sample was used to eliminate the methylene stretches and thus enhancing the band at 3020 cm\(^{-1}\). The differential infrared absorbance value versus iodine value of Castor oil did not fall on the straight line which all the other vegetable oils fit. This was attributed to the inductive effects of the hydroxyl group in the predominant fatty acid, ricinoleic. Hydrogenated samples were also found to have absorbance versus iodine values that do not fall on the curve with the other oils. These results were not unexpected because the iodine value determined by the Wijs method measures both cis and trans double bonds, while in the IR method the trans =C-H stretch absorbance is low relative to that of the corresponding cis band, effectively giving a measure of the number of cis double bonds only. Hence, this IR method is unsuitable for the analysis of hydrogenated samples which contain high levels of trans components.

A slightly different approach was used by Mitteilungen (1973) to measure the cis =C-H stretching band absorbance. This involved running solution phase spectra, at known concentrations and pathlengths. The "tangent" baseline procedure was used in the calculation of extinction coefficient. The differential technique described above was also tried, but found to give unsatisfactory results. The extinction coefficients of the cis =C-H stretch were established for a number of unsaturated fatty acids and FAME's, and found to be proportional to the number of \(74\)
cis double bonds. Mitteilungen suggested that this technique could be used to determine the cis content, but gave no examples. Only one paper, by Yulina (1987), has described the use of an alternative cis band to establish the cis content. This band was the cis =C-H deformation at 720 cm\(^{-1}\) which overlaps considerably with the methylene rocking band. The present author is extremely sceptical about the feasibility of this IR method. In general, IR techniques for determining cis content have not achieved the same popularity as the IR techniques of establishing the trans content, probably because food researchers prefer GC techniques which provide more information concerning the quantitation of the particular cis components. No studies have been conducted using these IR techniques in other laboratories, so these methods will not be discussed in terms of the five criteria put forward in the introduction.

3.2.3 Analysis of fatty acids by gas chromatography

GC procedures for the analysis of fatty acid methyl esters are considered in this section, and this is discussed in terms of both packed and Wall Coated Open Tubular (WCOT) columns. Essentially the difference in performance between these two types of columns comes down to resolution; the WCOT columns having superb resolution which presents the analyst with the problem of identifying large numbers of minor components. Packed columns, on the other hand, provide simpler chromatograms, but usually with much of the essential information, making it considerably easier to identify and quantify the components. Before the advantages and disadvantages of the two types of column are discussed in more detail, the liquid phases used in a GC column will be commented upon, because this is the principle factor determining the nature of the separations that can be achieved.

Liquid phases can be subdivided into two groups, polar and non-polar. Non-polar silicone liquid phases, such as SE-30, OV-1, JXR or QF-1, permit the separation of FAME's mainly on the basis of chain length, when in packed columns. However, WCOT columns with these phases can also separate unsaturated FAME's of the same chain length. Apiezon grease is another non-polar phase, which consists of high molecular weight hydrocarbons, capable of separating saturated and unsaturated components of the same chain length on WCOT columns, including positional and geometrical isomers (the unsaturated esters eluting before the related saturated compounds). On packed columns there is very little separation of esters with the same chain-length differing in degree of unsaturation.
Polar phases can be subdivided into four main classes:

**Group a;** the highest polarity phases, e.g. alkylpolysiloxanes containing various polar substitutes including nitrile groups, such as Silar 10C, Silar 9CP, SP 2340 and OV-275.

**Group b;** highly polar phases, e.g. polyethyleneglycol succinate (EGS), polydiethyleneglycol succinate (DEGS), EGSS-X (a copolymer of EGS with methyl silicone), CP-Sil 84 and CP-Sil 88 (cyano propyl silanol).

**Group c;** Medium polarity phases, e.g. polyethyleneglycol adipate (PEGA), polybutanediol succinate (BDS) and EGSS-y (a copolymer of EGS with a higher proportion of the methylsilicone than in EGSS-X).

**Group d;** low polarity phases, such as polynopentylglycol succinate (NPGS), EGSP-Z (a copolymer of EGS and phenyl silicone), Carbowax 20M (polyglycol) and Silar 5CP (cyanosilicone).

On these polar phases the methyl esters of the unsaturated acids are eluted after the corresponding saturates. This is due to a polar interaction of the phase with the methyl esters of unsaturated acids (Litchfield, 1964). Thus, the greater the polarity of the column the greater the degree of separation achieved, e.g. on packed columns, phases in groups a and b are capable of separating corresponding cis and trans mono-enes isomers (order of elution - saturates, trans then cis), whereas those in groups c and d are not. On WCOT columns, most of the phases have been reported to separate cis and trans isomers, however only the phases in group a and b can separate positional isomers. An enormous number of papers have been published describing the applications of these phases to the analysis of fats and oils. It is beyond the scope of this review to attempt to give examples, instead the reader is referred to two reviews by Ackman (1986) and Jaeger et. al. (1981).

One of the major issues when the GC technique was first introduced concerned the rearrangement and loss of unsaturated FAME's under chromatographic conditions. Morris et al. (1960) were the first to conduct studies in this area. They established that the esters of conjugated trienoic acids underwent cis/trans isomerisation, and that unsaturated esters containing hydroxy groups adjacent to the double bond were dehydrated. Mikolajczak et al. (1964) observed double bond migration in methyl eleostearate during GC analysis. However, other investigations found the more common non-conjugated polyenoic esters, including
methyl arachidonate, to be essentially unchanged during GC (Schlenk et al., 1962; Schlenk et al., 1965). This problem was eventually attributed to two factors, the use of active support materials and trans-esterifications of methyl esters with the polyester liquid phase (caused by residues of the catalyst required for the preparation of the polyester). Hence, those components remaining on the column the longest suffered the greatest losses. These problems have been resolved by later workers. Bannon et al. (1987) have shown that polyunsaturated FAME's do not decompose at high temperatures (375 °C), even when injected into an insert containing copper particles with hydrogen as the carried gas.

Identification of the individual components on a chromatogram has traditionally involved the instinctive understanding of the relationship between the retention times of the peaks on a chromatogram and their identity. When a relatively simple fatty acid mixture (such as corn oil) is analysed for the first time, there should be no problem of identification, because its composition is well documented in the literature. However, problems of identification may arise when new samples are analysed, or when trace components are seen for the first time, as is common with samples analysed on capillary columns for the first time. In such cases, these components may be identified by direct comparison of the retention times with those of standard mixtures containing accurately known amounts of saturated, monoenoate, and polyenoate FAME's, analysed on the same column under identical conditions. Comparisons should ideally be made on at least two columns with phases of different polarity. Standard mixtures are readily available from various suppliers, e.g. cod liver oil which has been analysed by Melcalfe and Wang (1981).

Ideally, the gas chromatographic retention time should not vary from one analysis to the next, and thus the retention time could be used to identify accurately a specific component of a mixture. Modern GC instruments give very reproducible chromatograms, however, factors such as column deterioration, carrier gas flow variation, injection technique, temperature program repeatability, and ambient temperature fluctuations can result in variations in retention times. Thus, the absolute retention time of an ester on any GC column has very little meaning as a measure of its elution characteristics. An alternative approach, involving the retention time of a fatty acid ester relative to that of a chosen commonly-occurring component (normally, 16 or 18), has been shown to be a better indicator of elution order, and thus more suited to inter-laboratory comparisons. For example the relative retention time ($r_{18:0}$) of an ester is its retention time divided by that of 18:0 ester. It should be noted that relative retention times vary with changes in the conditions outlined above, but these variations are comparatively small, and are in the same direction for all components.
The equivalent chain length (ECL), a concept first developed by Woodford and Van Gent (1960) and by Miwa et al. (1960), is generally a more acceptable and convenient mode of expressing the retention properties of a FAME, allowing its position in a chromatogram to be readily visualised in relationship to nearby saturated FAME's. Furthermore, peaks due to unknown FAME's can be tentatively identified. ECL is defined according to equation 3.2.2.1.

\[ \text{ECL} = n + \frac{\log t'_{R,x} - \log t'_{R,n}}{\log t'_{R,n+1} - \log t'_{R,n}} \]

- \( t'_{R,x} \) = correction retention time of unknown FAME \( x \)
- \( t'_{R,n} \) = corrected retention time of nearest saturated FAME eluting ahead of \( x \);
- \( t'_{R,n+1} \) = corrected retention time of next higher homologue of \( n \);
- \( t'_R = t_R - t_M \), where \( t_R \) = uncorrected retention time; and \( t_M \) = column dead time.

Studies of ECL data for cis and trans monoethylenic FAME's, and methylene-interrupted diethylenic FAME's isomers, on various liquid phases on WCOT columns have been reviewed by Christie (1989) and Conacher (1976). These studies are significant because the unsaturated FAME's that were used resemble those present in hydrogenated fish oils and provide the analyst with an indication of the potential separation that can be achieved. More importantly, they show which isomers will coelute with other isomers or saturated FAME's. In addition, ECL data can be used to predict the retention time of a FAME, or establish the identity of an unknown (Christie, 1989). Examples of the applications of ECL data will be given in the following sections.

It should be made clear that ECL values from a particular laboratories under specified conditions can rarely be reproduced in another. Nevertheless, while the specific numerical values have no absolute significance, the order of elution of particular components does have considerable significance.

3.2.3.1 Packed columns

Polar phases are used almost universally for fats and oils analysis, due primarily to the inability of non-polar packed columns to separate saturated and unsaturated components of the same
chain length. Two classes of moderately polar stationary phases have been commonly used in fatty acid methyl esters studies, polyesters and cyanosilicones. These polyesters such as diethylene glycol succinates (DEGS), EGS, EGA and butanediol succinate (BDS) were introduced in the late 1950s (Orr et al., 1958; Craig, 1959). The SP-2300 series cyanosilicones were introduced in the period 1973 to 1974 having separating characteristics similar to the polyesters, but having the capacity for longer column life. Both these classes of packing materials achieve separation on the basis of both chain length and degree of unsaturation, and are therefore unsuitable for the study of geometrical or positional isomers.

Separation of geometrical isomers was not achieved on packed columns until the introduction of highly polar cyanopropyl silicones stationary phases such as Silar 10C and SP-2340 (Heckers et al., 1977; Scholfield, 1979). These phases necessitated the use of long columns up to 6m in length. Witting et al. (1984) found that better resolution of cis and trans isomers could be obtained on the more polar dicyanoallyl silicone (OV-275) and established that the improved resolution between the geometrical isomers was due to the increased nitrile content of this phase. A 6 m x 2 mm (i.d.) column containing 15% OV-275 is used in the AOCS official Method cd 17-85, to determine the cis and trans isomers content. On these polar phases, saturated FAME's are eluted first followed by the trans then the cis isomers. This method is suitable for hydrogenated vegetable oils, where the major trans acid component is 18:1Δt and only small quantities of 18:2Δtt were present. Unfortunately, this column is unable to separate the complex mixtures of isomers present in hydrogenated fish oils. This is because a broad spectrum of cis and trans positional isomers are present, resulting in broadening and overlap of the cis and trans GC peaks which thus affect quantitation (Ottenstein et al., 1977). Hydrogenated fish oils are extensively used in margarines sold in Europe, but are not permitted in the USA where this packed column method was developed and hence the analytical problem does not arise. Although the method cannot cope with complex isomer mixtures, it does offer certain advantages over the newer capillary GC. These advantages include, ease of use for those inexperienced in capillary GC and ease of interpreting the results because there is no separation of the positional isomers.

An alternative method of improving the packed column GC separation of geometrical isomers is derivatisation. This involves the conversion of the unsaturated FAME's into one of the following derivatives; vinyl bromides, cyclopropanes, dihydroxy compounds and epoxides (Conacher, 1976). The most promising technique in this category is the epoxidation procedure which has achieved cis/trans separation of 18:1Δ monoenees with a wide spread of double bond positions. 

79
(Boniforti et al., 1985). However, this procedure does have two drawbacks, a lengthy derivatisation procedure and overlapping of the trans-15,16-epoxystearate and the main cis-epoxystearate peaks (Gunstone et al., 1972). This suggests that in complex mixtures of isomers, such as hydrogenated fish oils, resolution between all the isomers may not be possible.

A summary of the five criteria is presented below for packed columns coated with OV-275 phase;

**Specificity:** The following FAME's can be separated, saturates, trans monoenes, cis monoenes and polyenes. Unfortunately, cis and trans monoenes and polyenes positional isomers of the same chain length overlap, making these packed columns unsuitable for the analysis of hydrogenated fish oils, because they possess a wide distribution of these isomers.

**Sensitivity:** Levels below 1% of an individual component can easily be determined using an flame ionisation detector (FID). The only problem is overlap of components.

**Accuracy:** Results obtained using model mixtures of methyl elaidate and methyl oleate showed good agreement between actual weight percent trans and that calculated from peak areas. The relative percent deviation among all values varied from 1% to 10%, with the error increasing as the percent trans approached low values (Perkins et al., 1977). No accuracy studies have been conducted on hydrogenated fish or vegetable oil, but the difference between the known and established values would be expected to be greater, due to the overlap of the isomers.

**Precision:** No publications have been found describing repeatability results. Reproducibility using these packed columns has been found to be better than the traditional IR procedure in a large collaborative trial (Gildenberg et al., 1985).

**Robustness:** Procedures using highly polar packed columns are not likely to be as robust as IR procedures because they have a larger number of variables; variation of liquid phase from column to column and batch manufactured, and decomposition of the liquid phase with time.

### 3.2.3.2 WCOT columns

As in the case of packed columns, the analysis of oils and fats on WCOT columns are almost exclusively conducted on polar phases, although the resolution of WCOT columns is such that
some remarkable separations can be achieved with non-polar phases, which are both more stable and cheaper than polar phases. In fact, the separation of methyl oleate and methyl elaidate on capillary columns was first described for non-polar columns such as Apieson L. ECL data for some cis and trans methyl octadecenoate positional isomers on this column has been produced by Barve et al. (1972). This study showed that separations are possible for the Δ5-Δ10 cis isomers from the trans isomers. However, cis isomers possessing double bonds further from the carboxyl than Δ10 eluted with trans isomers. Analysis of fats and oils with a narrow range of position isomers can be analysed successfully according to these results. Unfortunately these non-polar columns suffer from long retention times and are unable to separate important fatty acids such as methyl linoleate and methyl linolenate (Ackman, 1986; Piretti et al., 1987). As these FAME's are essential fatty acids having major nutritional importance, the deficiency in this aspect of the separation has prejudiced the wider use of non-polar column. Having said this, it should be noted that remarkable separations can still be achieved with such columns. For example, a 44 m column coated with the non-polar OV-73 has been shown to achieve the separation of hydrogenated fish oil, rivalling the separation obtained with polar phases (Spark et al., 1982). Non-polar phases do have advantages in specific applications due to their stability at high temperatures, e.g. their use in GC-mass spectroscopy, where their inertness and low rates of bleed are virtues, and in the study of high molecular weight FAME's. Since the non-polar columns have inherent problems separating essential fatty acids, the polar columns have been almost universally accepted for the analysis of lipids. This is reflected by the vast numbers of publications which describe the use of these columns (Jaeger et al., 1981).

ECL data have been tabulated for a number of synthetic positional/ geometrical isomers of C18 unsaturated FAME's (monoenes, dienes and polyenes) on various polar columns (see reviews by Actman, 1986; Christie, 1989). The elution orders (or ECL values) of these positional isomers on polar columns have been explained theoretically in terms of the shapes of the molecules and the probability of interaction between the double bond and the liquid phase (Beaumelle et al., 1986). On these columns, cis and trans monoenes with the double bond in the same position are easily separated. The ECL values tend to increase with the distance of the double bond from the carboxyl group, and the degree of separation has been found to increase with polarity of the column. Increasing column length also improves the resolution between corresponding cis and trans isomers, but to a lesser degree. In general, 50 m columns are favoured over the 100 m columns, because they offer a faster analysis at the expense of a small loss in resolution. The use of shorter columns has also been described (Lercker, 1983; Sampugna et al., 1982). For example, the latter workers used a 15 m SP 2340 column to establish the cis and trans content of
lipids, incorporating a factor to compensate for the incomplete resolution of these isomers.

Unfortunately, even on the 100 m highly polar column, not all the positional isomers of monoenes are separated. For example, trans monoenes with double bonds at high $\Delta$ positions overlap with cis isomers having the double bond at low $\Delta$ positions. In the case of dienoic and polyenoic fatty acids the theoretical number of positional and geometrical isomers are immense. Certain unsaturated fatty acids, such as linoleic and linolenic, are predominantly found in natural products, except in the case of fish oils which contain significant quantities of other polyunsaturated fatty acids. Christie (1989) has reviewed the ECL data published for a number of polyunsaturated FAME's on various capillary columns, illustrating the overlap problems encountered between polyunsaturated FAME's and other classes of FAME's.

Analysis of oils and fats from animal and vegetable sources are easily achieved on polar capillary columns, however, this is not true for hydrogenated vegetable, and in particular hydrogenated fish oils, because of the greater distribution of positional and geometrical isomer present (see section 1.1.5). A number of authors have described the analysis of hydrogenated vegetable oils on polar columns (Supelco Bulletin, 1986; Ottenstein et al., 1984; Takagi et al., 1984; Bohov et al., 1984; Sampugna et al., 1982; Ackman et al., 1974). Regrettably, questions must be asked about the validity of the quantitative results obtained using these columns, because of the extent of overlap observed in the chromatograms produced. Similar views have been expressed by Van Vleet et al. (1978) and Strocchi et al. (1984), claiming that many workers have exaggerated the quality of separation that can be achieved on polar columns. Furthermore, they suggest that better results are obtained on long packed columns of OV-275, as this column separate only total cis-isomers from total trans-isomers for a given carbon chain length and will not separate any positional isomers. Hydrogenated fish oils contain a wider spread of isomers and of chain lengths, consequently chromatograms of these oils exhibit considerable overlap of cis and trans peaks. Hence, the determination of these FAME's cannot be achieved by this technique alone, notwithstanding claims made by several workers (Ackman et al., 1978; Ojanpera, 1978; Sisfontes et al., 1981).

Reproducibility of quantitative results produced by capillary GC techniques in various laboratories is notoriously poor. This is primarily due to the absence of a standard method of capillary GC analysis, which means that a variety of stationary phases, columns and conditions are used by different laboratories, making it impossible to obtain reproducible results. An additional factor which contributes to poor reproducibility and repeatability, involves the injection technique, in particular split injection to capillary columns. This commonly used technique is known to cause
needle discrimination. Grob (1986) has reviewed in great detail split and splitless injection in capillary GC, including procedures to optimise results and eliminate discrimination. A less detailed procedure of reducing needle discrimination in the analysis of FAME’s has been described by Bannon et al. (1987).

Beaumelle et al. (1986) have shown that FAME’s varying in chain-length and degree of unsaturation have different response factors (RF) in flame ionisation detectors and that these differences may be of primary importance in quantitative analysis. Badings et al. (1983) have recommended that each laboratory should calculate its own RF values (using standards and GC conditions identical to those used in the analysis), because RF values are influenced by various factors which makes them instrument as well as laboratory dependent. In general corrections for RF values are not used by lipid chemists.

Only WCOT column coated with the most polar phases will be discussed below in terms of the five criteria described in the introduction;

Specificity: These columns are capable of separating saturates, positional/geometrical monoene and polyene isomers of the same chain length. Unfortunately, peaks due to some positional trans-monoene isomers overlap with the peaks of cis isomers of the same chain length. Consequently, these columns are unsuitable for hydrogenated fish oils.

Sensitivity: Slover et al. (1979) have reported detecting peaks corresponding to 50-150 picograms of FAME on the column, or 5-15 nanograms injected using a split injection system and an FID detector.

Accuracy: Lanser et al. (1988) reported results for standard mixtures of methyl elaidate and methyl oleate (% w/w methyl elaidate). The relative percent deviation among all values varied from -0.5% to 8.7%, with the error increasing as the percent trans approached low levels. Slover et al. (1979) conducted studies on two reference mixtures and found the summed deviations of the results to be 2.43% and 3.06%. The compositions of these reference mixtures consisted of saturates, monoенes and polyenes, however, no positional isomers were present. No studies of samples containing a complex distribution of positional isomers have been conducted, but the results would be expected to show large differences from the known values on account of peak overlap.

Precision: Various publications have reported repeatability studies (Slovene et. al., 1979; Bannon et al., 1987; Athnasios et al., 1986; Muskiet et al., 1998). In general, the composition of
the fats and oils studied did not have a large distribution of saturated or unsaturated FAME's. The coefficient of variation for the different FAME's varied between 0.1% and 15%, the highest values were generally obtained for those FAME's which were not adequately resolved (e.g. positional isomers). Factors such as poor injection technique and deterioration of the stationary phase have been shown to reduce repeatability considerably. Only one reproducibility study has been reported in which good agreement was obtained between the laboratories, but this must be attributed to the simple composition of the mixture used in the study. Reproducibility must be one of the key issues regarding capillary column GC procedures, on account of the lack of a standard method. The variety of stationary phases, columns and conditions being used make it difficult for different laboratories to obtain reproducible results with more complex mixtures.

Robustness; A high degree of skill and experience is required by the analyst to obtain repeatable results and optimum separation, making this technique extremely unrobust.

3.3 EXPERIMENTAL

3.3.1 Preparation of the calibration solutions

For Trans FAME's; two methods of preparing the standards solutions were used;

(a) Stock solutions of FAME's were prepared by accurately weighing a known amount of the appropriate FAME (=0.5g) into a 50ml volumetric flask and diluting with tetrachloromethane (CCl₄), unless otherwise stated. The calibration solutions were prepared by transferring aliquots of the stock solution into 25ml volumetric flasks which were diluted to volume with the appropriate solvent.

(b) The calibration solutions were prepared by accurately weighing the FAME into separate volumetric flasks and diluting with the corresponding solvent.

The concentration range of the calibration solutions covered 1x10⁻³ to approximately 45x10⁻³ moles per litre for all trans FAME's.
For cis FAME's; cis FAME's calibration solutions were prepared according to procedure (a) described above. The concentration range of the calibration solutions was 0.013 to 0.135 moles per litre.

For two-component calibration solutions; known aliquots from stock solutions of the appropriate FAME's used in the two-component solutions were transferred into volumetric flasks and diluted to volume with tetrachloromethane.

3.3.2 Scanning conditions

The conditions used throughout this chapter to obtain the solvent and solution spectra were identical to those described in section 2.3.2, unless otherwise stated. The liquid cells were cleaned between successive scans by rinsing several times with the solution (or solvent) to be scanned next.

3.3.3 Subtraction of solvent spectrum from solution spectra

The subtraction of the solvent spectra from the solution spectra, to produce the resultant solute spectra, was achieved using either of the two OBEX programs SUBA1.oy and SUBA3.oy (details of these programs can be found in section Appendix II). Both programs use the difference routine provided in the CDS package. The latter routine converts the solvent and solution spectra from transmittance to absorbance before taking the difference between the ordinate values of the two spectra at each data point. The difference calculation for each point is;

\[ M_3 = M_1 - (M_2 \times F) \]

Where M3 represents the ordinate value of the difference (solute) spectrum, M1 represents the ordinate value of the solution spectrum, M2 represents the ordinate value of the solvent spectrum, and F is a normalisation factor applied to the pure solvent spectrum to make it equal to the solvent component of the solution spectrum. Thus, when the normalised solvent spectrum
is subtracted from the spectrum of the solution, the spectrum of the solute should remain.

The normalisation factor can be determined by one of the following methods:
(a) Since the pathlengths are the same for both the solution and solvent spectra a normalisation factor of one can be assumed.
(b) The computer can be used to determine the normalisation factor by selecting a band common to both the solution and solvent spectra, but not the solute. The normalisation is computed as follows;

\[ \frac{(\text{MAX}_{M_1} - \text{MIN}_{M_1})}{(\text{MAX}_{M_2} - \text{MAX}_{M_2})} = F \]

Where MAX and MIN are ordinate absorbance values on either side of a common band present in the solution (\(M_1\)) and solvent (\(M_2\)) spectra. Thus the object is to reduce the common band to zero and therefore normalise both spectra for variations.

Method (a) was used throughout this chapter, whereas, method (b) was used only in section 3.4.1 (selection of solvent). The latter method is more complex and details of the bands used to determine the normalisation factor are described in the following passages. Figure 3.3.3.1 and 3.3.3.2 show the spectra of chloroform and tetrachloromethane superimposed on the spectrum of methyl elaidate, respectively. They clearly show that there are a number of bands present in each solvent spectrum which are not present in the spectrum of methyl elaidate. From these bands the following were considered suitable for the determination of the normalisation factor by method (b).

**Chloroform**
- band a: 900 to 864 cm\(^{-1}\)
- band b: 2574 to 2300 cm\(^{-1}\)

**Tetrachloromethane**
- band c: 1650 to 1475 cm\(^{-1}\)
- band d: 649.5 to 608 cm\(^{-1}\)
3.3.4 Determination of the peak height absorbance using the straight baseline correction technique

This method was used to determine the peak height absorbance of a band using a straight baseline to correct for overlapping bands. A specially written OBEY program called Absorbpr.oy was used for this task. Details of this program can be found in Appendix II. The method was used to determine the corrected absorbance of various cis and trans bands. Of these, the trans $\text{–C-H}$ deformation is the most important with respect to this project, and it has been chosen to illustrate how the program works.

The Absorbpr.oy program functions by measuring the trans peak absorbance (OA) at N2 (see figure 3.3.4.1) and the absorbance of the baseline tangent (OB) which is produced by drawing a line from N1 to N3 and then subtracting the absorbance of the tangent from the absorbance of the trans peak to obtain the baseline corrected absorbance (AB). The wavenumbers N1, N2 and N3 used by the program to determine the trans absorbance values were kept constant for each set of resultant spectra produced by each of the subtraction methods described in section 3.3.3, unless otherwise stated. These parameters are manually selected by the analyst.

3.3.5 Determination of the peak height absorbance using the curved baseline simulated (CBS) correction technique

This method was primarily used to establish the cis $\text{C-C}$ stretching vibration band peak height absorbance ($\approx 1654 \text{ cm}^{-1}$), which lies on the shoulder of the strongly absorbing carbonyl band ($\approx 1742 \text{ cm}^{-1}$). To speed up the various spectral manipulations and calculations required to achieve the task, a number of OBEY programs were written. The first of these programs was called SIM1.oy, but it underwent various evolutionary changes to form the latest SIM4.oy program, details of which can be found in Appendix II. All these programs use the spectrum of methyl stearate in the region 1700 to 1600 cm$^{-1}$ to simulate the curved baseline which represents the carbonyl shoulder. This is achieved by multiplying the spectrum of methyl stearate by a factor established by equation 3.3.5.1.

\begin{equation}
M = A_1/A_2
\end{equation}
Abbreviations used in equation 3.3.5.1;

\[ M = \text{multiplication factor used to multiply the methyl stearate (i.e. baseline) spectrum.} \]
\[ A_1 = \text{absorbance value at 1700 cm}^{-1} \text{ in sample spectrum with cis C=C stretching band.} \]
\[ A_2 = \text{absorbance value at 1700 cm}^{-1} \text{ in methyl stearate spectrum being used to simulate the curved baseline.} \]

The absorbance value at 1700 cm\(^{-1}\) was found empirically to be the best wavenumber to establish the multiplication factor. After the methyl stearate spectrum had been multiplied by the factor, the corrected absorbance of the peak was calculated by subtracting the baseline absorbance (\(A_4\)) from the peak absorbance (\(A_3\)) as indicated in figure 3.3.5.1. Alternatively, the multiplied spectrum of methyl stearate could be subtracted from that of the solute to produce the resultant spectrum. This could be either plotted, printed or stored on a floppy disc.

In the latest program SIM4.oy, instead of using just one spectrum of methyl stearate in the cis C=C band region, a selection of 12 spectra of methyl stearate at various concentrations are available. This was found to be necessary due to a change in profile of the carbonyl shoulder with concentration (see section 3.4.7.4). A BASIC program (COM1.ba) incorporated into the SIM4.oy program selects the best fitting methyl stearate spectra after multiplication, by determining which spectrum has the least difference at a certain wavenumber (1620 cm\(^{-1}\), selected empirically).

3.3.6 Integrated peak area as a measure of the peak absorbance

Peak area determination was accomplished using the Obey program AREA.oy which was written for the task (see Appendix II). This program recalls a set of spectra sequentially and calculates the area between two wavenumber limits, as illustrated in figure 3.3.6.1, which are selected by the analyst. The spectra used for analysis may be in either absorbance or transmittance, while the area is always calculated in absorbance x cm\(^{-1}\).

When determining the trans peak area the solute spectra were used, therefore eliminating the solvent component from the results. In the case of the cis C=C stretching band, the spectra had
the solvent component subtracted (using the SUBA3.oy program) followed by the elimination of the carbonyl shoulder (using the CBS correction technique).

3.3.7 Smoothing and flattening of spectra

The solvent and corresponding solution spectra were smoothed and flattened using the CDS programs provided by Perkin Elmer prior to the subtraction process. The smooth routine applies the Savitzky/Golay smoothing function to the spectral data (parameter used S13), whilst the flattening procedure manipulates the spectrum to produce an approximately level baseline. It should be noted that the spectra were only smoothed and flattened where stated.

3.3.8 Determination of the extinction coefficient of cis and trans bands (using reference standard FAME's)

The extinction coefficient (k) of various cis and trans bands were established by obtaining the spectra of a set of calibration solutions of the corresponding FAME of interest using tetrachloromethane as the solvent and a liquid cell of known pathlength. The conditions used to obtain the spectra and the baseline correction technique/parameters will be stated in each section. The k is established by dividing the slope of the straight line Beer's law plot (peak absorbance [baseline corrected] versus concentration) by the cell thickness in centimetres (Hannah, 1974a). Thus;

\[ k = \frac{A}{(M \times L)} \]

\( k \) = Extinction coefficient of band as a function of concentration  
(concentration either as grams per litre or moles per litre)  
\( A \) = Gradient of Beer-Lambert plot  
\( M \) = Concentration either as grams per litre or moles per litre  
\( L \) = Sample path length in cm (inside cell thickness). Determined according to the procedure described by Hannah et al. (1974b)

Note: a least squares regression program which forces the line through the origin was utilised to establish the gradient of the plot.
3.3.9 Demountable cell

A description of the demountable cell (with spacers) used to obtain the spectra and the procedure applied to fill the cell has been given by Hannah et al. (1974c).

3.3.10 Methylation methods used on fats and oils

The following techniques were used to methylate the fats and oils studied.

3.3.10.1 Methanolic potassium hydroxide methylation method

The method described by Bannon et al. (1985) was used, with modifications: approximately 200 mg of the sample was accurately weighed into a stopped container of approximately 10 ml capacity and then dissolved in hexane (2 ml). 2N methanolic potassium hydroxide (100 μl) was added, the mixture shaken vigorously for thirty seconds and then allowed to react for 30 minutes at room temperature. The reaction was stopped by adding 2N HCl with shaking, until neutral. Aqueous and organic layers were then allowed to separate before the procedure described in section 3.3.12 was followed to prepare the FAME sample solution used.

3.3.10.2 Tetramethyl ammonium hydroxide (TMAH) methylation method

This is based on a method described by Wang et al. (1981) and was used throughout this project to methylate the samples of fats and oils, unless otherwise stated. The fat or oil sample (300 mg) was placed in a 10 ml screw cap vial and dissolved in diethyl ether (∼4 ml). To this, 0.3 ml of 20% (w/w) TMAH in methanol was added using a syringe. The vial was capped and then shaken for 1 to 2 minutes before water saturated with sodium chloride (∼3 ml) was added. After shaking the two layers were allowed to separate. The procedure describing the preparation of the FAME solutions of fats or oils samples is given in section 3.3.12.
3.3.11 TLC optimisation of methylation methods

The modifications to the methylation methods described in sections 3.3.10.1 and 3.3.10.2 were a direct result of a series of TLC experiments which were used to measure the extent of methylation under various conditions. The extent of methylation was established using reference fat N88, by spotting the top organic layer (≈ 1μl) from the methylation experiment onto a TLC plate (silica gel G-60, Schleicher & Schnell). Solutions of methyl stearate and the sample (pre-methylation) were also spotted onto the plate to establish the positions of the FAME’s and triglycerides. The TLC plate was developed by placing in a tank containing the solvent mixture; petroleum ether 40-60: diethyl ether: acetic acid in the ratio 90:10:1 (volume to volume). Spots were visualised using iodine and assessed against the FAME and triglyceride standards.

3.3.12 Preparation of FAME solutions from the samples of fats and oils

The organic layer containing the methyl esters from the methylation experiments were separated from the aqueous layer and dried by filtering through filter paper containing dried MgSO₄. The remaining aqueous layer was washed several times with the corresponding organic solvent, which was then dried and combined with the original organic solvent fraction. The solvent was evaporated off under reduced pressure. Approximately 200 mg of sample was accurately weighed into a volumetric flask (5ml) and diluted to volume with tetrachloromethane.

3.3.13 Determination of the cis or trans content by IR

Listed below are the equations used in the respective regression programs to determine the cis and trans content of samples.

\[ Y = MX \]

\[ \text{Trans or cis % content} = \frac{F(A \times 100)}{(M \times W)} \]
b. Least squares linear regression \((Y = MX + C)\)

\[
\text{Trans or cis \% content} = \frac{F((A - C) \times 100)}{(M \times W)}
\]

c. Quadratic regression \((Y = aX^2 + bX + c)\)

\[
\text{Trans or cis \% content} = \frac{100F\left(\left(\frac{(A - \frac{4ac - b^2}{4a})}{a} - \frac{b}{2a}\right)\right)}{W}
\]

Where:-

\[A = \text{Absorbance of band (peak height or peak area) of band of interest.}\]

\[M = \text{Gradient of calibration plot}\]

\[C = \text{Intercept of linear calibration plot}\]

\[a, b, c = \text{Coefficients of quadratic equation}\]

\[W = \text{Weight of sample (grams)}\]

\[F = \text{Dilution factor (volume in which weight of sample is dissolved divided by 1000)}\]

Note: The calibration spectra used to generate the calibration plots were produced using identical subtraction, absorbance determination procedures and experimental conditions to those utilised to produce the sample spectra and absorbance values. All the calibration plots were generated by plotting concentration of FAME (grams/litre) versus absorbance.

### 3.4 RESULTS AND DISCUSSIONS

From the assessment of current IR methods used to determine the trans content of fats and oils (see section 3.2.1), evaluated using various criteria (specificity etc.), two importance issues became apparent. These are;

a. The ability of the methods to obtain accurate and precise results depends upon the baseline correction technique used. This is particularly true for the analysis of samples containing low trans levels.

b. No comprehensive quantitative studies have been conducted on trans FAME's of different chain lengths or positional isomers. Therefore, the influence of the molecular weight distribution
of FAME's in a mixture on the accuracy of the results, which are calculated in terms of the percent weight of methyl elaidate, is unknown.

Similar issue became apparent for the current IR techniques used to determine the cis content. In sections 3.4.5 and 3.4.6 investigations of the two issues (a) and (b) are reported for the methods used by the author to establish the cis and trans content of fats and oils. In addition, new quantitative IR methods used to establish the cis content utilising alternative bands and baseline techniques are described in sections 3.4.7 and 3.4.8. The selection of the solvent used, optimisation of scanning conditions and solvent component subtraction will now be discussed.

3.4.1 Selection of solvent for solution phase studies of FAME's and solvent component subtraction technique

At the present time the solvent most commonly used in IR methods for determining the trans content of fats and oils is carbon disulphide (CS$_2$). This is on account of its transparency in the region of the trans peak, allowing baseline subtraction to be performed easily, and a large pathlength (0.2 mm to 2 mm) cell to be used. CS$_2$ has a number of disadvantages such as its toxicity, low boiling point and noxious odour. These factors make it totally unsuitable for the analyses of large numbers of samples.

The FTIR technique offers the potential of spectral manipulation, widening the choice of solvents which can be used in the solution phase study of FAME's. Three solvents were considered suitable, chloroform, tetrachloromethane and bromoform. However, the high cost of small quantities of FAME's and small wavenumber shifts of bands in the different solvents restricted the studies of FAME's to one solvent. The choice of this solvent was dependent upon two considerations; the ability of the subtraction method to remove interfering solvent bands in the trans region and the transparency of the solvent in other spectral regions of interest. These regions included the carbon hydrogen stretching region (3150 to 2700 cm$^{-1}$), the C=C stretch region (1700 to 1600 cm$^{-1}$) and any of the finger print region (1450 to 1100 cm$^{-1}$).

The first solvent studies to be conducted involved six methyl elaidate solutions in chloroform (see Table 3.4.1.1). Spectra were produced using standard conditions (see Table 3.4.1.5) and a
0.05 mm pathlength cell. This pathlength was chosen because it gives rise to only two solvent spectral bands which approach zero transmittance (see figure 3.3.3.1). The trans band, carbon hydrogen stretching and most of the finger print regions are clearly visible in the solute spectrum. Three different solvent component subtraction parameters were used to establish the best method of subtraction (see section 3.3.3). One of these involved the subtraction of the solvent spectrum from the solution spectrum using a normalisation factor of 1, while the other two involved the use of bands only present in the solvent to establish the normalisation factor (band a = 900 to 864 cm\(^{-1}\) and band b = 2574 to 2300 cm\(^{-1}\)). Poor linearity was obtained for the calibration plots of the trans band absorbance (straight baseline corrected) versus methyl elaidate concentration using this pathlength cell, irrespective of the solvent component subtraction technique used (see Table 3.4.1.5). This was primarily due to noisy resultant solute spectra. Increasing the cell pathlength 0.05 mm to 0.2 mm dramatically reduced the noise, allowing good linear plots of trans peak absorbance versus methyl elaidate concentration to be obtained down to 3x10\(^{-3}\) moles/litre using any of three solvent subtraction techniques (see Table 3.4.1.5). The trans absorbance values obtained using the three subtraction techniques were very similar, and any differences may be attributed to the use of different baseline parameters (see Table 3.4.1.1). Comparison of the R squared and intercept values (which are a measure of the goodness of fit) of the calibration plots using the three different subtraction techniques and the 0.2 mm cell in Table 3.4.1.5, reveals that the plot obtained using the normalisation factor of 1 to subtract the solvent component has the best fit. All the following solution phase studies in this chapter were conducted using a normalisation factor of 1.

A major problem with CHCl\(_3\) is the presence of a number of bands in the spectrum which absorb strongly and approach zero transmission. These bands cannot be subtracted, thus the resultant solute spectra are not observed in these regions and hence prevent the study of other bands present in the solution spectra of FAME's. This problem increases as the pathlength of the cell becomes larger. Figure 3.4.1.1 shows the spectrum of CHCl\(_3\) taken with a 0.2 mm cell and illustrates the regions where the resultant solute spectra are not observed.

Table 3.4.1.2 shows the concentration range of methyl elaidate solutions in bromoform and the trans peak absorbance values (baseline corrected) obtained using a 0.5 mm cell. The resultant solute spectra in the trans region showed less noise than the corresponding spectra in chloroform (0.2 mm cell). This was reflected in the calibration plot regression data (see Table 3.4.1.5) and is partly attributed to the greater transparency of bromoform in this region (see figure 3.4.1.2) and also due to the use of a larger pathlength cell. Bromoform was not selected for
general use because it is considerably more expensive than either of the other two solvents and offers few advantages over chloroform.

Table 3.4.1.1; Calibration data of methyl elaidate in chloroform solutions using various subtraction methods (see section 3.3.3);

<table>
<thead>
<tr>
<th>conc. mol/l x10⁻³</th>
<th>trans peak absorbance x10⁻³ (baseline corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05 mm cell</td>
</tr>
<tr>
<td></td>
<td>a</td>
</tr>
<tr>
<td>3.47</td>
<td>2.91</td>
</tr>
<tr>
<td>6.95</td>
<td>5.03</td>
</tr>
<tr>
<td>10.42</td>
<td>5.18</td>
</tr>
<tr>
<td>13.9</td>
<td>6.62</td>
</tr>
<tr>
<td>17.4</td>
<td>6.82</td>
</tr>
<tr>
<td>34.74</td>
<td>15.91</td>
</tr>
</tbody>
</table>

Where;

a = band a
(baseline parameters; N1 = 998 cm⁻¹, N2 = 970 cm⁻¹, N3 = 944 cm⁻¹)

b = band b
(baseline parameters; N1 = 998 cm⁻¹, N2 = 970 cm⁻¹, N3 = 944 cm⁻¹)

c = normalisation factor of 1
(baseline parameters; for 0.05 mm cell - N1=998 cm⁻¹, N2=970 cm⁻¹, N3=954 cm⁻¹; For 0.2 mm cell - N1 = 998 cm⁻¹, N2 = 970 cm⁻¹, N3 = 946 cm⁻¹)

Tetrachloromethane offered the largest spectral windows in the resultant solute spectra, allowing most of the methyl elaidate spectrum to be observed, even when a cell of 0.5 mm pathlength was used (see figure 3.4.1.3). It does have one disadvantage, namely a strong absorbance in the region of the trans band. The implications of this are that larger errors may be expected in the region of the trans band (e.g. noisier resultant spectra). Table 4.3.1.3 shows the concentration range of methyl elaidate solutions (dissolved in CCl₄) used, and the corresponding trans peak heights (baseline corrected) obtained from the spectra using a 0.2 mm pathlength cell. The R squared value and intercept were not as good as those from the plot obtained using CHCl₃ and a
0.2 mm cell, on account of noisy resultant solute spectra obtained at lower concentrations (see figure 3.4.1.4). The applications of smoothing and flattening functions (see section 3.3.7) to the solvent and solution spectra before the subtraction process dramatically reduced the noise (see figure 3.4.1.5), but did not improve the R squared value or the intercept of the Beer-Lambert plot obtained with this data (not included in tables). Further studies applying these functions were therefore discontinued. An obvious method of reducing noise is to increase the number of scans. However, the number used (50) was already quite large, and any worthwhile increase would have led to problems of heating of the solution by the IR beam. The remaining option was an increase in cell pathlength to 0.5 mm.

Table 3.4.1.2; Calibration data of methyl elaidate in bromoform solutions, (normalisation factor for subtraction = 1, parameters used to establish baseline; N1 = 998 cm\(^{-1}\) N2 = 970 cm\(^{-1}\) N3 = 928 cm\(^{-1}\)

<table>
<thead>
<tr>
<th>sol.</th>
<th>conc. of solution mol/l</th>
<th>trans peak absorbance baseline corrected</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>0.00812</td>
<td>.0464</td>
</tr>
<tr>
<td>b.</td>
<td>0.01625</td>
<td>.09194</td>
</tr>
<tr>
<td>c.</td>
<td>0.02437</td>
<td>.13647</td>
</tr>
<tr>
<td>d.</td>
<td>0.0349</td>
<td>.19367</td>
</tr>
<tr>
<td>e.</td>
<td>0.04874</td>
<td>.29032</td>
</tr>
<tr>
<td>f.</td>
<td>0.08123</td>
<td>.45198</td>
</tr>
</tbody>
</table>

The trans peak absorbance values (baseline corrected) of the methyl elaidate solutions, dissolved in CCl\(_4\), re-assayed using the 0.5 mm cell are shown in Table 3.4.1.3. The R squared value of the Beer-Lambert plot produced from the data obtained using the 0.5 mm cell was better than that obtained using the 0.2 mm cell, however, the intercept value was not (see Table 3.4.1.5) and the resultant solute spectra of the three lowest solutions were still very noisy. Examination of the resultant solute spectrum of the lowest concentration solution of methyl elaidate (figure 3.4.1.6) revealed that the solvent spectrum was not correctly subtracted and interference fringes were also present, thus contributing to the error. Verification of this was
established in the methyl elaidate solution stability study, in which the solutions used above were stored in a dark cupboard for a week and re-assayed using a 0.5 mm cell, through which air was passed for approximately five minutes by means of a vacuum. This caused frosting of the cell windows and so reduced the interference fringes. In addition, the number of scans was increased from 5 to 10 scans. These measures considerably reduced the error in the solvent subtraction and to some extent the interference fringes as illustrated by solute spectrum of the solution with the lowest methyl elaidate concentration (figure 3.4.1.7). As expected, the \( R^2 \) squared and intercept of the Beer-Lambert plot were much improved (see Table 3.4.1.5).

A fresh set of methyl elaidate solutions dissolved in \( \text{CCI}_4 \) was prepared (see Table 3.4.1.4) and assayed using a 0.5 mm pathlength cell (frosted according to the procedure described above) and scanned using 5 and 10 accumulated scans. The results obtained confirmed those in the previous paragraph (see Table 3.4.1.5).

Studies using a 1 mm pathlength cell and the solutions described above were conducted in order to establish whether errors in the solvent component subtraction could be further improved and still allow all the other bands present in FAME's to be studied. Figure 3.4.1.8 represents the spectrum of \( \text{CCI}_4 \) obtained with a 1 mm cell. Only three bands approach zero transmittance between 4000 and 400 cm\(^{-1}\), therefore there are three regions in which solute spectra cannot be observed. Two of these regions (see figure 3.4.1.3) also approaches zero transmittance in the spectrum obtained using a 0.5 mm cell, while the other lies in a region in which no FAME's bands are present. Thus, the use of a 1 mm cell has no disadvantages compared with a 0.5 mm cell, with respect to the study of FAME bands not present in the trans band region. Figure 3.4.1.9 shows the liquid phase and the resultant solute spectra (obtained using a 1 mm cell) of methyl elaidate (region 1500 to 900 cm\(^{-1}\)), illustrating the extent to which the solvent component can be eliminated in this region.

Table 3.4.1.4 shows the trans peak absorbance values of the above solutions using a 1 mm cell. Two solvent spectra were used on the solution spectra in order to establish the influence of the solvent spectrum, one taken before all the solution spectra were taken and the other after all the solution spectra were taken. The trans peak absorbance values obtained using the two different solvent spectra were slightly different (see Table 3.4.1.4), however, the gradient and \( R^2 \) squared values obtained from the plots (Table 3.4.1.5) were almost identical. The intercept only differed slightly.

The resultant spectra obtained using the 1 mm cell showed the lowest noise and produced the
best plots. It can be concluded that the optimum pathlength for the scanning conditions is 1 mm. The problem associated with the quality of the solvent spectra (variation associated with the use of different solvent spectra, same cell) were reduced by increasing the number of scans from 10 to 50 in latter experiments. This was also found to improve the intercept value.

To summarise, CHCl₃ and CHBr₃ were not selected on account of their limited spectral windows, which effectively only allowed the study of the trans band. CCl₄ was selected as the solvent for the solution study of FAME's because it allowed all the spectral bands of interest in the spectra of FAME's to be studied using one solvent. The best results were obtained using a normalisation factor of 1 to subtract the solvent from the solution spectra, 50 scans accumulated and a cell of 1 mm pathlength with the standard scanning conditions.

Table 3.4.1.3; Calibration data of methyl elaidate in tetrachloromethane solutions

<table>
<thead>
<tr>
<th>conc. mol/l x10⁻³</th>
<th>trans peak absorbance x10⁻³ (baseline corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.2 mm cell (50 scans)</td>
</tr>
<tr>
<td>1.37</td>
<td>1.92</td>
</tr>
<tr>
<td>2.74</td>
<td>4.86</td>
</tr>
<tr>
<td>4.11</td>
<td>7.5</td>
</tr>
<tr>
<td>5.47</td>
<td>10.35</td>
</tr>
<tr>
<td>6.84</td>
<td>13.31</td>
</tr>
<tr>
<td>10.95</td>
<td>26.6</td>
</tr>
<tr>
<td>13.69</td>
<td>33.69</td>
</tr>
<tr>
<td>34.21</td>
<td>80.01</td>
</tr>
</tbody>
</table>

Parameters used to establish baseline;
0.2 mm cell - N₁ = 995 cm⁻¹ N₂ = 969 cm⁻¹ N₃ = 944 cm⁻¹.
0.5 mm cell (5 scans) - N₁ = 987 cm⁻¹ N₂ = 969 cm⁻¹ N₃ = 940 cm⁻¹.
0.5 mm cell (10 scans) - N₁ = 995 cm⁻¹ N₂ = 969 cm⁻¹ N₃ = 944 cm⁻¹.
* = spectra taken one week later.
(normalisation factor of 1 was used to subtract the solvent).
Table 3.4.1.4; Calibration data of methyl elaidate in tetrachloromethane solutions

<table>
<thead>
<tr>
<th>conc. mol/l x10^-3</th>
<th>trans peak absorbance (baseline corrected)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.5 mm cell</td>
</tr>
<tr>
<td></td>
<td>(5 scans)</td>
</tr>
<tr>
<td>2.00</td>
<td>0.01401</td>
</tr>
<tr>
<td>4.01</td>
<td>0.02651</td>
</tr>
<tr>
<td>8.01</td>
<td>0.05137</td>
</tr>
<tr>
<td>12.02</td>
<td>0.07631</td>
</tr>
<tr>
<td>16.03</td>
<td>0.10292</td>
</tr>
<tr>
<td>32.05</td>
<td>0.2053</td>
</tr>
<tr>
<td>40.07</td>
<td>0.26517</td>
</tr>
</tbody>
</table>

Parameters used to establish baseline;

N1 = 995 cm⁻¹, N2 = 969 cm⁻¹, N3 = 928 cm⁻¹, normalisation factor of 1 was used to subtract the solvent.

Where, a = solvent spectrum used was taken before all the calibration solution spectra were taken, b = solvent spectrum used was taken after all the calibration solution spectra were taken.
Table 3.4.1.5; Beer-Lambert plots data of methyl elaidate in various solvent and using several different pathlength cells and conditions.

<table>
<thead>
<tr>
<th>Table</th>
<th>solvent</th>
<th>pathlength of cell</th>
<th>factor</th>
<th>no. of scans</th>
<th>calibration plot</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gradient</td>
</tr>
<tr>
<td>3.4.1</td>
<td>CHBr₃</td>
<td>0.5 mm</td>
<td>1</td>
<td>50</td>
<td>5.61515</td>
</tr>
<tr>
<td>3.4.2</td>
<td>CHCl₃</td>
<td>0.05 mm band a</td>
<td>10</td>
<td></td>
<td>0.40226</td>
</tr>
<tr>
<td>3.4.2</td>
<td>CHCl₃</td>
<td>0.05 mm band b</td>
<td>10</td>
<td></td>
<td>0.40351</td>
</tr>
<tr>
<td>3.4.2</td>
<td>CHCl₃</td>
<td>0.05 mm</td>
<td>10</td>
<td></td>
<td>0.28231</td>
</tr>
<tr>
<td>3.4.2</td>
<td>CHCl₃</td>
<td>0.2 mm band a</td>
<td>10</td>
<td></td>
<td>1.979376</td>
</tr>
<tr>
<td>3.4.2</td>
<td>CHCl₃</td>
<td>0.2 mm band b</td>
<td>10</td>
<td></td>
<td>1.966298</td>
</tr>
<tr>
<td>3.4.2</td>
<td>CHCl₃</td>
<td>0.2 mm</td>
<td>10</td>
<td></td>
<td>1.96027</td>
</tr>
<tr>
<td>3.4.3</td>
<td>CCl₄</td>
<td>0.2 mm</td>
<td>1</td>
<td>50</td>
<td>2.415624</td>
</tr>
<tr>
<td>3.4.3</td>
<td>CCl₄</td>
<td>0.5 mm</td>
<td>1</td>
<td>5</td>
<td>6.184888</td>
</tr>
<tr>
<td>3.4.3</td>
<td>CCl₄</td>
<td>0.5 mm</td>
<td>10</td>
<td></td>
<td>6.558879</td>
</tr>
<tr>
<td>3.4.4</td>
<td>CCl₄</td>
<td>0.5 mm</td>
<td>1</td>
<td>5</td>
<td>6.501567</td>
</tr>
<tr>
<td>3.4.4</td>
<td>CCl₄</td>
<td>0.5 mm</td>
<td>10</td>
<td></td>
<td>6.471715</td>
</tr>
<tr>
<td>3.4.4</td>
<td>CCl₄</td>
<td>1.0 mm</td>
<td>1</td>
<td>10a</td>
<td>12.7102</td>
</tr>
<tr>
<td>3.4.4</td>
<td>CCl₄</td>
<td>1.0 mm</td>
<td>1</td>
<td>10b</td>
<td>12.71</td>
</tr>
</tbody>
</table>

Where - factor = normalisation function used
3.4.2 Stability of methyl elaidate in CCl₄

Isomerisation of double bonds from cis to trans and from trans to cis during hydrogenation is well documented (see section 1.1.5), however, whether isomerisation or decomposition of the double bond of FAME's in CCl₄ solutions occurs has not been established. To investigate this problem an experiment was conducted in which methyl elaidate solutions dissolved in CCl₄ were assayed and the trans peak absorbance (baseline corrected) determined. These solutions were kept in the dark for a week at room temperature and reassayed. Table 3.4.2.1 shows results for the most concentrated solutions studied. Corrections were applied because the pathlengths of the cells used were not identical.

<table>
<thead>
<tr>
<th>Sample</th>
<th>trans peak absorbance (baseline corrected)</th>
<th>pathlength of cell</th>
<th>corrected absorbance</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A)</td>
<td>0.21506</td>
<td>0.521</td>
<td>0.413</td>
</tr>
<tr>
<td>(B)</td>
<td>0.22519</td>
<td>0.5376</td>
<td>0.419</td>
</tr>
</tbody>
</table>

These results indicate that isomerisation or decomposition of the trans double bond of methyl elaidate has not occurred in CCl₄ while stored in the dark for a week. The differences are within experimental error. It should be noted that isomerisation is more than likely to occur in the presence of light.

3.4.3 Extinction coefficients of the trans band (baseline corrected) of various trans FAME's

All the IR techniques used to establish the trans content of fats and oils use the quantitative measurement of the trans band at approximately 969 cm⁻¹ and report the results in terms of
percentage weight of methyl elaidate. Naturally questions have been asked as to whether the other trans FAME's, varying in chainlength or the position of the trans double bond along the chain, have similar absorbance values for the trans band and if they do not, what effect this would have upon the calculated results.

Table 3.4.3.1: Extinction coefficient of the trans band of various FAME's reported in the literature (k as a function of concentration {grams/litre}).

<table>
<thead>
<tr>
<th>Samples</th>
<th>Extinction coefficient</th>
<th>reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:1Δ6t</td>
<td>0.454</td>
<td>Shreve et al. (1950)</td>
</tr>
<tr>
<td>18:1Δ9t</td>
<td>0.442</td>
<td>Shreve et al. (1950)</td>
</tr>
<tr>
<td>18:1Δ6t</td>
<td>0.456</td>
<td>Swern et al. (1950)</td>
</tr>
<tr>
<td>18:1Δ9t</td>
<td>0.442</td>
<td>Swern et al. (1950)</td>
</tr>
<tr>
<td>18:1Δ6t</td>
<td>0.456</td>
<td>Shreve et al. (1950)</td>
</tr>
<tr>
<td>18:1Δ9t</td>
<td>0.552</td>
<td>Shreve et al. (1950)</td>
</tr>
<tr>
<td>Acids</td>
<td>18:1Δ6t</td>
<td>Swern et al. (1950)</td>
</tr>
<tr>
<td></td>
<td>0.560</td>
<td>Swern et al. (1950)</td>
</tr>
<tr>
<td></td>
<td>0.552</td>
<td>Shreve et al. (1950)</td>
</tr>
<tr>
<td></td>
<td>0.552</td>
<td>Shreve et al. (1950)</td>
</tr>
<tr>
<td></td>
<td>0.601</td>
<td>Ahlers et al. (1953)</td>
</tr>
<tr>
<td></td>
<td>18:1Δ9t12(OH)</td>
<td>Ahlers et al. (1953)</td>
</tr>
<tr>
<td></td>
<td>0.547</td>
<td>Ahlers et al. (1953)</td>
</tr>
<tr>
<td></td>
<td>18:2Δ9t12t</td>
<td>Ahlers et al. (1953)</td>
</tr>
<tr>
<td></td>
<td>0.912</td>
<td>Ahlers et al. (1953)</td>
</tr>
<tr>
<td></td>
<td>18:3Δ9t12t15t</td>
<td>Ahlers et al. (1953)</td>
</tr>
</tbody>
</table>

Very little data has been published on the extinction coefficient of the trans band of FAME's or acids, see Table 3.4.3.1. The published data was obtained using the null method to determine the trans peak absorbance, and carbon disulphide was the solvent. A study was therefore conducted by the author to establish the influence of changing the chain length of the fatty acid chain, or position of the trans double bond, on the extinction coefficient of the trans band. Table 3.4.3.2 shows the trans band extinction coefficient, in terms of grams per litre (k2) and moles per litre (k1), for various trans FAME's using different pathlength cells. The extinction coefficients (k) were obtained using the spectra of calibration solutions (CCl4 solvent) with a normalisation factor of one to subtract the solvent component. The same straight baseline parameters were applied to determine the trans peak height absorbance (N1=995 cm⁻¹, N2= trans peak, N3= 944 cm⁻¹, unless otherwise stated). The regression program traversing zero intercept was utilised to establish the gradient of the calibration plot (see section 3.3.8).
The results obtained generally show that FAME's with only one trans double bond have $k_1$ values which increase as the chain length increases, but only by a small amount (see plot 3.4.3.1). Exceptions to this include 18:1$\Delta$9t12(OH), deuterated methyl elaidate (18:1$\Delta$9t(d3)) and 18:1$\Delta$11t. In the case of 18:1$\Delta$9t12(OH) the lowering of the $k_1$ value is caused by the overlap of the hydroxyl band with the trans band. This is illustrated by figure 3.4.3.1, which shows how the increase in the absorbance at N1 results in a reduction in the trans peak absorbance and thus a lower $k$ value. The lower $k_1$ value of methyl-d$_3$-elaidate was attributed to the differences in contribution from the overlapping bands which shift position when the methoxyl group is deuterated (see figure 3.4.3.2). In the case of 18:1$\Delta$11t the lower than expected $k_1$ value was possibly due to a weighing error. Verification of this was provided by the extinction coefficient value of the carbonyl band (559) which was significantly lower than that of other monoene trans FAME's (577 to 580).

A totally different picture is obtained when the $k_2$ values of the various FAME's are compared (see plot 3.4.3.2 and Table 3.4.3.2). Here the lower molecular weight (MWT) FAME's have higher values than the higher molecular weight FAME's with the same number of trans double bonds. This is attributable to the larger quantity of low MWT FAME's per gram. Very little difference was observed between the $k_2$ values of the two positional isomers 18:1$\Delta$6t and 18:1$\Delta$9t, however the value of 18:1$\Delta$11t was significantly lower, but this has been explained in the previous paragraph. This suggests that the $k_2$ values of positional isomers between $\Delta$6 and $\Delta$9 are very similar, unfortunately there is still uncertainty about the values between $\Delta$9 and $\Delta$11.

The implications of the above for fats and oils with a high percentage of only one trans FAME, such as 14:1$\Delta$9t, is that the trans content will be ca.16% higher than the true value on account of the form in which the results are expressed; the percentage weight of methyl elaidate. Likewise, in a sample containing only 22:1$\Delta$13t, the trans content value will be ca. 14% lower than the true value. In practice, fats and oils containing a high percentage of trans content have, in general, a distribution range for the carbon number of trans monoenes between C12 and C24, the greatest proportion of trans FAME's being C18 isomers. Thus the errors are likely to partially cancel one another.

Both the $k_1$ and $k_2$ values of methyl linoleadate and methyl trans-11,14-seicosadienoate, which both have two isolated double bonds, were expected to be twice the respective $k$ values of methyl elaidate. However, both were found to have $k$ values which were approximately one and a half to one and three quarters the corresponding values of methyl elaidate. Ahlers et al. (1954)
also observed that the k of linoelaidic acid (18:2Δ9t12t) was not twice that of elaidic acid but attributed this to the low purity of the sample used. They also found that trans trans conjugated 9,11-linoelaidic had an k value twice that of elaidic acid. These results suggest that considerable errors in the trans content will be obtained when high levels of trans conjugated and non-conjugated polyenes are present in samples. This factor was recognised at an early stage in the development of trans IR methods, consequently, most of the methods stipulate that they are applicable to the determination of isolated trans bonds in samples which contain only small amounts (less than 5%) of conjugated fatty acids.

Studies on methyl elaidate dissolved in CHCl₃ and CHBr₃ showed the corresponding extinction coefficients of the trans band to be significantly lower than that of methyl elaidate in CCl₄ (see Table 3.4.3.2). This is presumed to be due to interactions between the solvent and the FAME. Quantitative solution studies of the trans band were also conducted on Methyl-d₃-elaidate. This was conducted because the methoxyl group bands, which are known to overlap with the trans band and cause baseline error, are deuterated and shifted to a lower wavenumber. The k₁ and k₂ values obtained were lower than the corresponding values of methyl elaidate, which suggests that the straight baseline correction technique did not eliminate all of the absorbance of the interfering overlapping bands and thus errors are still present. Although two different sets of baseline parameters were used to establish the corrected trans peak absorbance of methyl elaidate and methyl-d₃-elaidate, this was not considered to be the reason for the differences in the k values.

From these results it can be concluded that for samples with trans components, consisting mainly of monoenes with a distribution range of carbon numbers C16 to C20, and positional isomers Δ6 to Δ11, the level of error in the IR trans content calculated will be low. Samples containing high levels of trans polyenes and trans monoenes FAME's (outside the distribution range Δ6 to Δ11) will produce a repeatable error in the trans content, which may be higher or lower than the true value depending upon the composition.
Table 3.4.3.2; The extinction coefficient of the trans band of various trans FAME's and the position of the band.

<table>
<thead>
<tr>
<th>FAME</th>
<th>Pathlength (mm)</th>
<th>$k_1$ (cm$^{-1}$)</th>
<th>$k_2$ (cm$^{-1}$)</th>
<th>Trans peak position (cm$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:1Δ9t</td>
<td>1.005</td>
<td>119 (0.94)</td>
<td>0.495 (1.16)</td>
<td>969</td>
</tr>
<tr>
<td>16:1Δ9t</td>
<td>1.005</td>
<td>120 (0.95)</td>
<td>0.447 (1.04)</td>
<td>968</td>
</tr>
<tr>
<td></td>
<td>0.506</td>
<td>122 (0.96)</td>
<td>0.454 (1.09)</td>
<td>968</td>
</tr>
<tr>
<td>18:1Δ6t</td>
<td>1.005</td>
<td>126 (0.99)</td>
<td>0.425 (0.99)</td>
<td>969</td>
</tr>
<tr>
<td></td>
<td>0.512</td>
<td>126 (0.99)</td>
<td>0.425 (0.99)</td>
<td>969</td>
</tr>
<tr>
<td>18:1Δ9t</td>
<td>1.009</td>
<td>127 (1)</td>
<td>0.428 (1)</td>
<td>969</td>
</tr>
<tr>
<td></td>
<td>1.005</td>
<td>127 (1)</td>
<td>0.428 (1)</td>
<td>969</td>
</tr>
<tr>
<td></td>
<td>1.005</td>
<td>127 (1)</td>
<td>0.428 (1)</td>
<td>969</td>
</tr>
<tr>
<td></td>
<td>1.009</td>
<td>127 (1)</td>
<td>0.428 (1)</td>
<td>969</td>
</tr>
<tr>
<td>18:1Δ9t (CHCl$_3$)</td>
<td>0.521</td>
<td>101 (0.8)</td>
<td>0.341 (0.8)</td>
<td>970</td>
</tr>
<tr>
<td>18:1Δ9t (CHBr$_3$)</td>
<td>0.521</td>
<td>107 (0.84)</td>
<td>0.360 (0.84)</td>
<td>970</td>
</tr>
<tr>
<td>18:1Δ9t (d$_3$)</td>
<td>1.005</td>
<td>103 (0.81)</td>
<td>0.344 (0.8)</td>
<td>969</td>
</tr>
<tr>
<td>18:2Δ9t11t</td>
<td>1.005</td>
<td>115 (0.91)</td>
<td>0.388 (0.91)</td>
<td>969</td>
</tr>
<tr>
<td>18:1Δ9t12(OH)</td>
<td>1.005</td>
<td>106 (0.83)</td>
<td>0.339 (0.79)</td>
<td>973</td>
</tr>
<tr>
<td>20:1Δ11t</td>
<td>1.005</td>
<td>129 (1.02)</td>
<td>0.398 (0.93)</td>
<td>969</td>
</tr>
<tr>
<td>20:2Δ11t14t</td>
<td>1.005</td>
<td>205 (1.61)</td>
<td>0.636 (1.5)</td>
<td>970</td>
</tr>
<tr>
<td>22:1Δ13t</td>
<td>1.005</td>
<td>131 (1.03)</td>
<td>0.370 (0.86)</td>
<td>969</td>
</tr>
</tbody>
</table>

Where; $k_1$ = The extinction coefficient as a function of concentration (moles per litre).

$k_2$ = The extinction coefficient as a function of concentration (grams per litre). Values in brackets represent ratio of corresponding FAME $k$ value divided by value of methyl elaidate.

18:1Δ9t (CHCl$_3$) = methyl elaidate solutions dissolved in CHCl$_3$

18:1Δ9t (CHBr$_3$) = methyl elaidate solutions dissolved in CHBr$_3$

18:1Δ9t (d$_3$) = deuterated methyl elaidate
3.4.4 Determination of the trans % content of model mixtures using the peak height straight baseline correction technique

Extinction coefficient results from the methyl-d3·elaidate experiment described in the previous section suggested that the straight line baseline correction technique, used to calculate the corrected trans peak absorbance, does not fully compensate for the absorbance of the bands which overlap with the trans band.

To assess to what extent the selection of the baseline parameters influence the accuracy of the method, studies were conducted on model mixtures of methyl oleate and methyl elaidate. Two sets of wavenumber parameters (A) and (B) were used to determine the trans peak height of the model mixture spectra and the methyl elaidate calibration spectra. All the spectra were taken using the standard scanning conditions (50 co-added scans) and a 1 mm cell.

Wavenumber parameters

(A) \( N_1 = 995 \text{ cm}^{-1}, N_2 = 969 \text{ cm}^{-1}, N_3 = 944 \text{ cm}^{-1} \)

(B) \( N_1 = 981 \text{ cm}^{-1}, N_2 = 969 \text{ cm}^{-1}, N_3 = 950 \text{ cm}^{-1} \)

Table 3.4.4.1 shows the trans content values determined using the two wavenumber parameters (A) and (B). Both of these parameters gave values which were lower than the known values for the mixtures. However, for mixture (a), which contains the highest trans content, more accurate results were obtained using wavenumber parameters (A), whereas, for mixtures with low a trans content (i.e. mixtures (c) to (d)) wavenumber parameters (B) produced more accurate results. Clearly, neither set of baseline parameters copes with changes in the profile of the overlapping bands which occur when the percentage of cis content is changed. Figures 3.4.4.1 and 3.4.4.2 show the solute spectra of mixtures (a) and (e), which are the two most extreme cases, with the two baselines produced using the different wavenumber parameter sets. It is apparent from these figures that at high trans levels the wavenumber parameter set (A) produces the best baseline fit, whilst in mixtures of low trans content the reverse is true. The difference values in Table 3.4.4.1 confirm this.

Problems with the goodness of fit of the different wavenumber parameter sets were not encountered in the methyl elaidate calibration spectra, because the profile of the overlapping bands with the trans band remains constant regardless of concentration.
Table 3.4.4.1; trans % content of model mixture determined by IR method using two different sets of wavenumber parameters to establish the peak height. Where MO = methyl oleate, ME = methyl elaidate and values in bracket represent difference between known and determined values.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>composition % (w/w)</th>
<th>ME parameters (A)</th>
<th>ME parameters (B)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Known</td>
<td>MO</td>
<td>determined</td>
</tr>
<tr>
<td>(a)</td>
<td>62</td>
<td>38</td>
<td>60.1 (-1.9)</td>
</tr>
<tr>
<td>(b)</td>
<td>29</td>
<td>71</td>
<td>26.5 (-2.5)</td>
</tr>
<tr>
<td>(c)</td>
<td>17</td>
<td>83</td>
<td>14.4 (-2.6)</td>
</tr>
<tr>
<td>(d)</td>
<td>9.25</td>
<td>90.75</td>
<td>6.75 (-2.5)</td>
</tr>
<tr>
<td>(e)</td>
<td>2.5</td>
<td>97.5</td>
<td>-0.4 (-2.9)</td>
</tr>
</tbody>
</table>

Correcting for the absorbance of overlapping bands has been recognised by lipid analysts as a major problem with the IR methods used to determining the trans content. Two techniques have been adopted to overcome this problem; the first uses a solution of saturated FAME’s in the reference beam of the IR spectrometer to cancel out the interfering bands. In order for the technique to work well, the concentrations of saturated FAME and sample solutions must be equal. Furthermore, the composition, in terms of carbon chain-lengths, must also be the same for both solutions, because the interfering bands are attributed to the methoxy group, therefore the extinction coefficients would be expected to increase per gram with decrease in molecular weight of the FAME. If these conditions are not met then the subtraction of the interfering bands is expected to be either over or under compensated, producing an error. Examples of the application of this method in the literature have been largely limited to samples with 18 carbons in the fatty acid chain, i.e. closely resembling the reference sample composition. This technique was not used in the present work.

The second correction technique attempts to compensate for the interfering bands by using a two-component set of calibration solutions. Either a cis or saturated FAME can be used with methyl elaidate to make up these calibration solutions. This method assumes that the profiles of
the interfering bands are the same for both cis and saturated FAME's in the region of the trans band, regardless of chain-length. This technique will be discussed in detail in the sections 3.4.5.2 and 3.4.5.3.

Examination of the resultant solute spectra in the trans band region of methyl oleate and methyl stearate revealed that the band profiles are different (see figures 3.4.4.3 & 3.4.4.4). Greater spectral profile differences were observed for other cis FAME's varying in chain-length and degree of unsaturation. Questions must therefore be asked regarding the effect of the composition on the baseline, and therefore on trans peak height measurements. It should be noted that this effect is only going to be apparent in samples with low trans content.

Lancer et al. (1988) developed a method of determining the % trans content using the trans band area. They observed that the width of the trans band in a mixture decreases with concentration. To compensate for this they varied the wavenumber values of the baseline so that they matched the absorbance minimum on either side of the trans peak. Areas of the isolated trans absorbance band were calculated between the perpendicular lines dropped from the band minima to the baseline.

3.4.5 Determination of the trans content of various samples

3.4.5.1 Fat samples - B72E and a trial fat

The straight baseline technique described in the previous section was used to determine the trans content of two fats provided by the MAFF. Both sets of baseline parameters used in the previous section were applied to determine the trans peak absorbance values of the sample and the methyl elaidate calibration spectra. The data generated by FTIR, using different baseline parameters, were compared with the corresponding data obtained by capillary GC method to establish how well the different baseline compensate for the overlapping bands. The fat samples were methylated according to the procedure described in section 3.3.10.1, the methyl esters were then weighed and diluted for analysis by FTIR and capillary GC. Scanning and processing procedures for the FTIR were identical to those described in the previous section.
Table 3.4.5.1: Percentage of trans content (as a percentage weight of methyl elaidate (w/w)) in two fats determined by Capillary GC and FTIR methods.

| sample | conc.g/5ml | % trans content (w/w) | GC method | % unidentified |
|--------|------------|------------------------|-----------|----------------|----------------|
|        |            | IR method (A) | GC method (B) |                  |                |
|        |            | 41.5          | 41.5       | 40.8             | 0.1            |
| B72E   | 0.4023     | 41.5          | 41.5       | 40.8             | 0.1            |
| Trial fat | 0.2928 | 12.6          | 13.9       | 13.1             | 0.3            |

Where; wavenumber parameters used in baseline correction:

(A) \( N1 = 995 \text{ cm}^{-1} \), \( N2 = 969 \text{ cm}^{-1} \), \( N3 = 944 \text{ cm}^{-1} \)

(B) \( N1 = 981 \text{ cm}^{-1} \), \( N2 = 969 \text{ cm}^{-1} \), \( N3 = 950 \text{ cm}^{-1} \)

Very similar results were obtained by the capillary GC and FTIR methods for both fats. In the case of fat B72E, both sets of wavenumber parameters gave identical results, which were slightly higher than the GC result, whereas for the trial fat, the wavenumber parameters (B) gave a value that was higher than those produced by the other parameter set, and by the GC method. This agrees with the conclusions reached in the previous section, viz. that for mixtures containing less than 29% trans, wavenumber parameters (B) give a more accurate result. The results obtained for fat B72E are significant because both sets of wavenumber parameters gave the same result, suggesting that the errors in both sets of wavenumber parameters are equal when the mixture has 40% trans. It is probable that for samples containing more than 40% trans the wavenumber parameter set (A) produces the more accurate results, whereas, below 40% trans, set (B) is superior.

The GC results are generally lower than the FTIR results which are presumably more accurate. These differences could be due to the complexity of the isomers in the samples, and the difficulty in obtaining complete separation with the GC method. A more detailed account of the composition of these fats established by the GC method may be found in Table 3.4.7.9.1.
3.4.5.2 Repeatability of the % trans content by the capillary GC and FTIR methods and assessment of different calibration solutions

A standard fat (N88), provided by the MAFF, was used to establish the repeatability of the percentage trans content determined by capillary GC and FTIR. In addition to the methyl elaidate set of calibration solutions, a two-component set of calibration solutions of methyl stearate and methyl elaidate was used to determine the FTIR trans content results. Results generated using the two different calibration solutions were used to assess which of the two gives the best correction for the saturated and cis unsaturated absorbance in the trans region.

The composition of the two-component mixtures ranged from 2% to 30% trans. Eight such mixtures were made up according to the procedure described in section 3.3.1, so that the total concentration of both components equalled 40g/l. A total of nine repeat N88 fat methyl ester solutions were prepared following the methodology described in the previous section. Samples and calibration standards were scanned using a 1 mm cell and the standard procedure (50 co-added scans). A normalisation factor of one was used to subtract the solvent component. The two sets of wavenumber parameters described in the previous section were used to determine the trans peak height of the samples and of the calibration spectra. In addition, the trans peak height, without any baseline correction, was calculated for the samples and standards. These results were used to give an indication of the contribution from overlapping bands. Summarised below are the abbreviations and wavenumber parameters used in the straight baseline correction technique:

**Calibration solutions**

Method A = results generated using two-component (methyl stearate and methyl elaidate) calibration data (see Table 3.4.5.2.1)

Method B = results generated using single component (methyl elaidate) calibration data (see Table 3.4.5.2.1).

**Wavenumber parameters (WP) used to determine baseline**

(i) - No baseline parameters

(ii) - \( N_1 = 995 \text{ cm}^{-1}, N_2 = 969 \text{ cm}^{-1}, N_3 = 944 \text{ cm}^{-1} \)

(iii) - \( N_1 = 985 \text{ cm}^{-1}, N_2 = 969 \text{ cm}^{-1}, N_3 = 952 \text{ cm}^{-1} \)
Table 3.4.5.2.1; Determination of trans content by FTIR - least squares linear regression data for the two sets of calibration solutions using different baseline parameters (WP = wavenumber parameters).

<table>
<thead>
<tr>
<th>Method</th>
<th>WP</th>
<th>gradient</th>
<th>intercept</th>
<th>R²</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>(i)</td>
<td>0.044251</td>
<td>0.07125</td>
<td>0.999964</td>
</tr>
<tr>
<td>A</td>
<td>(ii)</td>
<td>0.041879</td>
<td>-0.06479</td>
<td>0.999964</td>
</tr>
<tr>
<td>A</td>
<td>(iii)</td>
<td>0.039826</td>
<td>-0.03644</td>
<td>0.999962</td>
</tr>
<tr>
<td>B</td>
<td>(i)</td>
<td>0.045789</td>
<td>0.01568</td>
<td>0.9999648</td>
</tr>
<tr>
<td>B</td>
<td>(ii)</td>
<td>0.040269</td>
<td>0.00876</td>
<td>0.999963</td>
</tr>
<tr>
<td>B</td>
<td>(iii)</td>
<td>0.038959</td>
<td>0.00946</td>
<td>0.999779</td>
</tr>
</tbody>
</table>

Table 3.4.5.2.2; FTIR % trans content repeatability results for fat N88 using two different sets of calibration data and various baseline parameters (1 mm cell).

<table>
<thead>
<tr>
<th>solution</th>
<th>trans content %</th>
<th>Method A</th>
<th>Method B</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(i)</td>
<td>(ii)</td>
<td>(iii)</td>
</tr>
<tr>
<td>a.</td>
<td>41.5</td>
<td>38.4</td>
<td>38.1</td>
</tr>
<tr>
<td>b.</td>
<td>40.6</td>
<td>40.8</td>
<td>39.6</td>
</tr>
<tr>
<td>c.</td>
<td>42.9</td>
<td>39.6</td>
<td>39.1</td>
</tr>
<tr>
<td>d.</td>
<td>43.3</td>
<td>41.4</td>
<td>40.4</td>
</tr>
<tr>
<td>e.</td>
<td>40.7</td>
<td>39.1</td>
<td>38.6</td>
</tr>
<tr>
<td>f.</td>
<td>41.8</td>
<td>39.6</td>
<td>39.2</td>
</tr>
<tr>
<td>g.</td>
<td>41.1</td>
<td>39.3</td>
<td>38.9</td>
</tr>
<tr>
<td>h.</td>
<td>41.8</td>
<td>39.2</td>
<td>39.0</td>
</tr>
<tr>
<td>i.</td>
<td>40.6</td>
<td>39.3</td>
<td>38.9</td>
</tr>
<tr>
<td>mean</td>
<td>41.6</td>
<td>39.6</td>
<td>39.1</td>
</tr>
<tr>
<td>std. dev.</td>
<td>1.0</td>
<td>0.9</td>
<td>0.7</td>
</tr>
<tr>
<td>C.V.</td>
<td>2.3%</td>
<td>2.3%</td>
<td>1.7%</td>
</tr>
</tbody>
</table>
Table 3.4.5.2.3; GC determined trans content of fat N88, repeatability experiment.

<table>
<thead>
<tr>
<th>Solution</th>
<th>% mono trans</th>
<th>% di trans</th>
<th>% cis/trans</th>
<th>unknown %</th>
<th>cal. IR trans %</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>32.7</td>
<td>0.61</td>
<td>1.09</td>
<td>2.82</td>
<td>35.0</td>
</tr>
<tr>
<td>b.</td>
<td>32.5</td>
<td>0.61</td>
<td>1.11</td>
<td>4.42</td>
<td>34.8</td>
</tr>
<tr>
<td>c.</td>
<td>33.3</td>
<td>0.61</td>
<td>1.13</td>
<td>3.53</td>
<td>35.6</td>
</tr>
<tr>
<td>d.</td>
<td>33.2</td>
<td>0.60</td>
<td>1.11</td>
<td>3.56</td>
<td>35.5</td>
</tr>
<tr>
<td>e.</td>
<td>33.9</td>
<td>0.59</td>
<td>1.12</td>
<td>3.74</td>
<td>36.5</td>
</tr>
<tr>
<td>f.</td>
<td>33.0</td>
<td>0.60</td>
<td>1.07</td>
<td>3.32</td>
<td>35.3</td>
</tr>
<tr>
<td>g.</td>
<td>34.0</td>
<td>0.61</td>
<td>1.14</td>
<td>3.60</td>
<td>36.4</td>
</tr>
<tr>
<td>h.</td>
<td>33.1</td>
<td>0.62</td>
<td>1.16</td>
<td>3.33</td>
<td>35.5</td>
</tr>
<tr>
<td>i.</td>
<td>33.1</td>
<td>0.59</td>
<td>1.10</td>
<td>4.14</td>
<td>35.4</td>
</tr>
<tr>
<td>j.</td>
<td>32.3</td>
<td>0.60</td>
<td>1.11</td>
<td>2.87</td>
<td>35.6</td>
</tr>
<tr>
<td>mean</td>
<td>33.1</td>
<td>0.60</td>
<td>1.11</td>
<td>3.53</td>
<td>35.5</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.6</td>
<td>0.01</td>
<td>0.03</td>
<td>0.50</td>
<td>0.5</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.7%</td>
<td>1.7%</td>
<td>2.7%</td>
<td>14.2%</td>
<td>1.3%</td>
</tr>
</tbody>
</table>

Notes: % mono trans = % mono trans determined by GC
% di trans = % di-unsaturated determined by GC
% cis/trans = % of cis/trans isolated di-unsaturated determined by GC
Unknown % = GC unidentified FAME %

The last column gives estimates of the "expected" total trans content determined by IR, according to the following equation. This equation takes into account the difference in absorbance associated with the degree of trans unsaturation.

\[
\text{cal. IR trans %} = \text{% mono trans} + \frac{\text{% cis/trans di}}{2} + 2(\text{% di trans})
\]
The results in Table 3.4.5.2.1 show the linear least squares data for the two sets of calibration solutions and the various wavenumber parameters used to determine the baseline correction. All the plots showed excellent adherence to Beer's law. Method B (ii) gave the smallest deviations from zero intercept.

Comparison of the IR and GC (either the % mono-trans or the GC estimated IR values) results for the trans content of fat N88 reveal that the largest differences were obtained using method A (i) and method B (i). This was as expected, because these IR methods were not baseline corrected, and thus had absorbance contributions from interfering bands associated with the saturated and cis-unsaturated FAME's. Two-component calibration data (method A) evidently provides some compensation for the interfering bands, because the trans value obtained (40.6%) is appreciably lower than that of the corresponding methyl elaidate calibration data (method B - 42.2%).

Mean values obtained using the IR methods B(ii) (36.5%) and B(iii) (36.9%) were very similar to each other and to the GC estimated IR value (35.5%). The latter takes into account the differences in absorption of the trans band with degree of unsaturation (see Table 3.4.5.2.3). Results using method B (ii) (39.6%) and B (iii) (39.1%) were significantly higher than the GC estimated IR trans content value. Two factors may account for these high values; the first is the relatively large combined percentage of minor unassigned FAME's (3.5%) in the chromatogram (figure 3.4.5.2.1). If all these unassigned FAME's were assumed to be trans, which is unlikely, then the total GC estimated IR trans content would be 39.1%. This value is almost the same as that obtained using the IR methods A(ii) and A(iii). Examination of the GC chromatogram of fat N88 reveals the second factor, poor resolution between the peaks. When these two factors are taken into account it seems likely that methods A(ii) and A(iii) provided the most accurate results.

With regard to repeatability, methods B(ii) and B(iii) had the best coefficients of variance; 1.6% and 1.5% respectively. It is interesting to note the the coefficient of variance of method A(iii) (1.7%) is significantly smaller than that of method A(ii) (2.3%); this may be attributed to the baseline parameters used. Although the precise composition of this fat is not known, the results obtained with the two-component calibration data were significantly higher than those using the single component calibration data. This suggests that the former is achieving better compensation for the interfering bands.
3.4.5.3 Comparison of two different two-component calibration data on the determination of the % trans content of various biscuit fats.

In the previous section, FTIR calibration data generated using a single (methyl elaidate) and a two-component (methyl elaidate and methyl stearate) set of calibration spectra, produced significantly different % trans values for a particular fat (N88). The two-component calibration results were considered to be the more accurate because the method compensated for the absorbance of the interfering bands.

Examination of the trans band region of methyl oleate (figure 3.4.4.4) and methyl stearate (figure 3.4.4.3) solution spectra revealed that the profiles of the overlapping bands differ. Comparisons of other FAME's in this region showed greater differences (see figures 3.4.5.3.1 to 3.4.5.3.4). This suggests that different % trans results may be obtained for the same sample using different two-component calibration data (i.e. methyl stearate/methyl elaidate or methyl oleate/methyl elaidate calibration data). Therefore, if the profile of the interfering bands do not resemble each other in both the sample and calibration spectra, an error will be introduced in the result. A series of biscuit fats standards, provided by the MAFF, varying in composition were assayed according to the FTIR methods described in the previous section. In addition to this, the % trans content was established using the calibration data generated from a set of methyl oleate/methyl elaidate solution spectra (using the same set of wavenumber baseline parameters for the baseline correction). All of these fats were also assayed by capillary GC. The objective of this work was to establish whether the conclusion stated in the previous paragraph is correct by comparing the results generated using different two-component calibration data.

Summarised below are the abbreviations used for the calibration solution sets and the wavenumber parameters:

**Calibration solutions used:**

- Methyl oleate/methyl elaidate - MO/ME
- Methyl stearate/methyl elaidate - MS/ME
- Methyl elaidate - ME

**Wavenumber parameters codes**

(i) $N1 = 995 \text{ cm}^{-1}$, $N2 = 969 \text{ cm}^{-1}$, $N3 = 944 \text{ cm}^{-1}$

(ii) $N1 = 985 \text{ cm}^{-1}$, $N2 = 969 \text{ cm}^{-1}$, $N3 = 952 \text{ cm}^{-1}$
Table 3.4.5.3.1: Calibration plot data of various sets of calibration solutions (WP = wavenumber parameters)

<table>
<thead>
<tr>
<th>solution</th>
<th>cell</th>
<th>WP</th>
<th>Calibration data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>gradient</td>
</tr>
<tr>
<td>MO/ME</td>
<td>1 mm</td>
<td>(i)</td>
<td>0.040254</td>
</tr>
<tr>
<td>MO/ME</td>
<td>1 mm</td>
<td>(ii)</td>
<td>0.038157</td>
</tr>
<tr>
<td>MS/ME</td>
<td>1 mm</td>
<td>(i)</td>
<td>0.042599</td>
</tr>
<tr>
<td>MS/ME</td>
<td>1 mm</td>
<td>(ii)</td>
<td>0.040368</td>
</tr>
<tr>
<td>ME</td>
<td>1 mm</td>
<td>(i)</td>
<td>0.045345</td>
</tr>
<tr>
<td>ME</td>
<td>1 mm</td>
<td>(ii)</td>
<td>0.044485</td>
</tr>
</tbody>
</table>

Table 3.4.5.3.2: Percentage trans content of various biscuit fats determined by FTIR (duplicate measurements were made on most samples).

<table>
<thead>
<tr>
<th>Biscuit fat</th>
<th>% trans content</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(i)</td>
<td>(ii)</td>
</tr>
<tr>
<td></td>
<td>MO/ME</td>
<td>MS/ME</td>
</tr>
<tr>
<td>7/15a</td>
<td>25.3</td>
<td>25.7</td>
</tr>
<tr>
<td>7/15b</td>
<td>25.2</td>
<td>25.6</td>
</tr>
<tr>
<td>11/19a</td>
<td>32.0</td>
<td>31.8</td>
</tr>
<tr>
<td>11/19b</td>
<td>32.9</td>
<td>32.8</td>
</tr>
<tr>
<td>4/6a</td>
<td>11.4</td>
<td>12.9</td>
</tr>
<tr>
<td>4/6b</td>
<td>11.8</td>
<td>13.0</td>
</tr>
<tr>
<td>9/10a</td>
<td>9.9</td>
<td>12.2</td>
</tr>
<tr>
<td>9/10b</td>
<td>10.0</td>
<td>12.4</td>
</tr>
<tr>
<td>13/17a</td>
<td>6.1</td>
<td>7.7</td>
</tr>
<tr>
<td>13/17b</td>
<td>6.1</td>
<td>7.6</td>
</tr>
<tr>
<td>5/14</td>
<td>16.6</td>
<td>17.4</td>
</tr>
<tr>
<td>3/8</td>
<td>-1.5</td>
<td>0.8</td>
</tr>
</tbody>
</table>
Table 3.4.5.3.3; GC analysis of the biscuit fats. Results are percentages.

<table>
<thead>
<tr>
<th>Biscuit fats</th>
<th>% of each FAME type</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>unid.</td>
<td>sat.</td>
<td>mono cis</td>
<td>mono trans</td>
<td>di- cis</td>
<td>di- trans</td>
<td>c/t</td>
<td>cis</td>
<td>IR</td>
</tr>
<tr>
<td>7/15a</td>
<td>6.2</td>
<td>28.3</td>
<td>34.9</td>
<td>23.6</td>
<td>4.7</td>
<td>0.5</td>
<td>0.7</td>
<td>1.2</td>
<td>-</td>
</tr>
<tr>
<td>7/15b</td>
<td>5.8</td>
<td>27.8</td>
<td>34.9</td>
<td>24.1</td>
<td>4.8</td>
<td>0.6</td>
<td>0.8</td>
<td>1.3</td>
<td>-</td>
</tr>
<tr>
<td>7/15 av.</td>
<td>6.0</td>
<td>28.1</td>
<td>34.9</td>
<td>23.8</td>
<td>4.7</td>
<td>0.5</td>
<td>0.8</td>
<td>1.3</td>
<td>25.7</td>
</tr>
<tr>
<td>11/19a</td>
<td>6.9</td>
<td>23.0</td>
<td>33.2</td>
<td>29.5</td>
<td>4.8</td>
<td>0.8</td>
<td>1.2</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>11/19b</td>
<td>6.3</td>
<td>23.4</td>
<td>33.3</td>
<td>29.5</td>
<td>4.7</td>
<td>0.8</td>
<td>1.2</td>
<td>0.9</td>
<td>-</td>
</tr>
<tr>
<td>11/19 av.</td>
<td>6.6</td>
<td>23.2</td>
<td>33.2</td>
<td>29.5</td>
<td>4.6</td>
<td>0.8</td>
<td>1.2</td>
<td>0.9</td>
<td>32.3</td>
</tr>
<tr>
<td>9/10a</td>
<td>1.4</td>
<td>43.9</td>
<td>31.4</td>
<td>11.4</td>
<td>11.2</td>
<td>0.0</td>
<td>0.3</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>9/10b</td>
<td>2.9</td>
<td>43.7</td>
<td>31.6</td>
<td>9.0</td>
<td>12.1</td>
<td>0.0</td>
<td>0.3</td>
<td>0.5</td>
<td>-</td>
</tr>
<tr>
<td>9/10 av.</td>
<td>2.2</td>
<td>43.8</td>
<td>31.5</td>
<td>10.2</td>
<td>11.7</td>
<td>0.0</td>
<td>0.3</td>
<td>0.5</td>
<td>10.2</td>
</tr>
<tr>
<td>4/6a</td>
<td>3.1</td>
<td>46.5</td>
<td>30.5</td>
<td>10.8</td>
<td>9.2</td>
<td>0.0</td>
<td>0.0</td>
<td>0.0</td>
<td>-</td>
</tr>
<tr>
<td>4/6b</td>
<td>5.3</td>
<td>44.5</td>
<td>30.4</td>
<td>10.6</td>
<td>8.9</td>
<td>0.0</td>
<td>0.2</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>4/6 av.</td>
<td>4.2</td>
<td>45.5</td>
<td>30.5</td>
<td>10.7</td>
<td>9.1</td>
<td>0.0</td>
<td>0.1</td>
<td>0.1</td>
<td>10.7</td>
</tr>
<tr>
<td>13/17</td>
<td>1.2</td>
<td>40.6</td>
<td>39.6</td>
<td>7.0</td>
<td>10.8</td>
<td>0.0</td>
<td>0.4</td>
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<td>7.4</td>
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<tr>
<td>5/14</td>
<td>10.5</td>
<td>41.0</td>
<td>31.7</td>
<td>8.0</td>
<td>8.0</td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
<td>8.4</td>
</tr>
<tr>
<td>3/8</td>
<td>2.0</td>
<td>79.7</td>
<td>9.3</td>
<td>1.7</td>
<td>6.9</td>
<td>0.0</td>
<td>0.0</td>
<td>0.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>

116
Abbreviations used in Table 3.4.5.3.3:-

unid. = unidentified FAME's
sat. = unsaturated FAME's
cal. IR = IR predicted trans content from GC results obtained in table
   = (GC mono trans %)+(GC di-c/t %)+2(GC di trans %)+3(GC tri trans %)
di-c/t = di-unsaturated cis/trans isomers
av. = average of two values

Good linear fits were obtained for all the calibration plots over the concentration ranges studied (see Table 3.4.5.3.1). Table 3.4.5.3.2 shows the FTIR % trans results for the fats. A summary of the composition of these fats established by capillary GC may be found in Table 3.4.5.3.3. Included in this table are the GC estimates of the IR % trans content (cal. IR), which will be used in the following paragraphs to compare the GC results with the corresponding IR results. The reason for doing this was explained in the previous section.

Biscuit fats 7/15 and 11/19 were established by the GC results to have the highest levels of trans FAME's (25.7% and 32.3%, respectively). The corresponding IR results for these fats, produced by both of the two-component calibration solutions, were very close to the GC estimated IR values, irrespective of the baseline parameters used. However, the values produced using the methyl elaidate calibration data were approximately 2-3% lower for both fats. The difference was slightly greater using baseline (I), as expected. These results confirm the conclusions stated in the previous section, that both the two-component calibration sets compensate successfully for the error introduced by the overlapping bands of saturated and cis-unsaturated FAME's. As no differences were observed between the IR results produced by the methyl oleate/methyl elaidate and methyl stearate/methyl elaidate calibration data, it can be said that the spectral differences of bands in the trans region of methyl oleate and methyl stearate are not significant. Using either methyl stearate or methyl oleate in the two-component calibration mixtures makes no difference to the results obtained when the trans content is in the range 25% - 32%.

Differences were observed between the results generated using the two sets of calibration data for the other fats. The differences can be attributed to the range of trans content being outside the 20% - 80% trans content range of the methyl oleate/methyl elaidate calibration solutions.
The IR results generated for biscuit fats 4/6, 9/10 and 13/17 using the methyl stearate/methyl elaidate two-component calibration solutions were higher than those generated using methyl elaidate alone. Both of these sets of IR results were higher than the GC estimated IR value, except in the case of fat 13/17 where the results were almost identical. The baseline parameters (i) tended to give lower values than the other parameters for the methyl elaidate calibration data. Surprisingly, the reverse was true for the methyl stearate/methyl elaidate results. The values obtained using methyl elaidate calibration data and the wavenumber parameters (i) were the closest to those of the GC estimated IR for fats 4/6 and 9/10. However, the percentage levels of unidentified FAME’s found by the GC method for fats 4/6 and 9/10 were 2.2% and 4.2% respectively. When this is taken into account the higher values obtained using ME parameter (ii) and MS/ME parameters (i) and (ii) seem more reasonable.

Biscuit fat 13/17 had the lowest levels of unidentified FAME’s by the GC method (1.2%) and therefore the most accurate GC estimated IR % trans content result. The results obtained using the ME and MS/ME calibration data confirm the above observation, i.e. that the % trans value of ME parameter (i) is low, while that of MS/ME (i) is high. Those of ME and MS/ME using parameter (ii) were very similar and most probably the most accurate.

Comparison of the GC estimated IR % trans content of fat 5/14 (8.4%) and the IR values (16 - 18%) caused some concern, until it was noted that this fat contained hydrogenated fish oil. Examination of the composition of the fat by GC shows that the level of unidentified FAME’s is extremely high (10.5%). Unfortunately, the capillary GC analysis of this fat was conducted at the MAFF laboratories, and all they provided was a summary of the composition similar to that shown in Table 3.4.5.2.3, therefore the extent of peak overlap could not be established. Fats 13/17 and 5/14 GC results were also provided by the MAFF, whereas, fats 7/15, 11/19, 4/6 and 9/10 were assayed by the author at Surrey University (note these fats were reassayed by the MAFF on their instruments and produced comparable results). If the % levels of unidentified FAME’s are added to the GC estimated IR % trans content, then a value approaching that produced by the IR methods is obtained. The results for fat 5/14 illustrate the inability of the GC technique to cope with complex mixtures of isomers. Very low values of trans content are likely to be produced.

Fat 3/8 according to the GC results contains high levels of saturated FAME’s (80%), low levels of trans (1.7%) and unidentified FAME’s (2.0%). Therefore the GC estimated IR value should be fairly accurate. IR results generated by the ME and MS/ME calibration data using parameter (i) were both significantly lower than the GC estimated IR value. The ME data, parameter (ii), produced the value (1.6%) with the smallest difference from the GC estimated value.
MS/ME parameter (ii) result was slightly lower (1.1%); this was attributed to the trans level being outside the calibration range of the solutions (2 to 40%).

From the preceding results the following can be concluded. The use of methyl oleate or methyl stearate with methyl elaidate in the two-component calibration solution has little or no effect upon the trans values obtained. However, this may not be true if different cis-unsaturated or saturated FAME's are used. The differences between the trans values obtained using the two sets of wavenumber parameters for the two-component calibration data were not as great as those observed for the methyl elaidate calibration data. Those values obtained from the two-component calibration data and the wavenumber parameters (ii) were considered to be the most accurate provided that the trans level was within the calibration range.

3.4.5.4 Comparison of % trans content obtained by peak height and peak area measurements

Four samples of fats, referred to as FT001, FT002, FT003 and FT004, were submitted by the MAFF to the author for trans content analysis using any method deemed appropriate. These samples were also submitted to a number of other laboratories with the same brief as part of a collaborative study. The composition of the fats were established by the MAFF using capillary GC, and were given to the author after the assays described below were performed, so that no bias could occur. Two results are shown for fat FT001, because the MAFF analysts reassayed this sample after seeing the large discrepancy between their original trans content results, and those obtained by the author using FTIR.

Five repeat samples of each fat were prepared after conversion to the methyl esters (see section 3.3.12 and 3.3.10.2). Spectra of these solutions were produced using the standard conditions and a 1 mm cell. The solute spectra were obtained by subtracting the solvent spectrum from the sample spectra. A normalisation factor of one was used.

Listed below are details of the various sets of calibration solutions used and the wavenumber parameters selected for the baseline. In addition to the peak height measurements, calibration data based on peak areas were also established, using an adapted version of the methods described by Sleeter et al. (1989) and Lanser et al. (1988). Linear least squares regression programs were used to determine the gradient and intercept of each calibration plot. These
were then used to determine the % trans content of the sample, by substituting the corresponding absorbance value (i.e. peak height or peak area values, determined using identical wavenumber parameters to those used on the calibration solution) into the equation described in section 3.3.13.

**Calibration solutions:**

Methyl stearate/methyl elaidate (MS/ME):- A total of 9 solutions in the range 2 to 30% trans content.

Methyl elaidate (ME):- Six solutions in the range 10 to 100 g/l.

Methyl oleate/methyl elaidate (MO/ME):- Six solutions in the range 30 to 95% trans content.

**Wavenumber parameters (WP)**

<table>
<thead>
<tr>
<th>Code</th>
<th>height/area</th>
<th>wavenumber parameters used</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i)</td>
<td>height</td>
<td>N1 = 995cm(^{-1}) (\text{N2} = 969cm(^{-1}) (\text{N3} = 944cm(^{-1})</td>
</tr>
<tr>
<td>(ii)</td>
<td>height</td>
<td>N1 = 965cm(^{-1}) (\text{N2} = 969cm(^{-1}) (\text{N3} = 952cm(^{-1})</td>
</tr>
<tr>
<td>(iii)</td>
<td>area</td>
<td>N1 = 976cm(^{-1}) (\text{N2} = 964cm(^{-1}) (\text{N3} = \text{varied})</td>
</tr>
<tr>
<td>(iv)*</td>
<td>height</td>
<td>N1 = varied (\text{N2} = 969cm(^{-1}) (\text{N3} = \text{varied})</td>
</tr>
<tr>
<td>(v)*</td>
<td>area</td>
<td>N1 = varied (\text{N2} = 976cm(^{-1}) (\text{N3} = 964cm(^{-1}) (\text{N3} = \text{varied})</td>
</tr>
</tbody>
</table>

* The last two types of techniques used to determine the absorbance values were only used on the calibration spectra of MS/ME and the sample spectra. "Varied" means the wavenumber was not kept constant, but changed to take into account the trans band broadening with increase in trans content.
Table 3.4.5.4.1; Calibration plots of trans content (g/l) vs. absorbance (peak height or area). The different baseline parameters are shown by (i) to (v).

<table>
<thead>
<tr>
<th>Set</th>
<th>height/area</th>
<th>code</th>
<th>calibration plot data</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>gradient</td>
</tr>
<tr>
<td>MS/ME</td>
<td>height</td>
<td>(i)</td>
<td>0.04279</td>
</tr>
<tr>
<td>MS/ME</td>
<td>height</td>
<td>(ii)</td>
<td>0.038281</td>
</tr>
<tr>
<td>MS/ME</td>
<td>area</td>
<td>(iii)</td>
<td>0.144788</td>
</tr>
<tr>
<td>MS/ME</td>
<td>height</td>
<td>(iv)</td>
<td>0.036585</td>
</tr>
<tr>
<td>MS/ME</td>
<td>area</td>
<td>(v)</td>
<td>0.33641</td>
</tr>
<tr>
<td>ME</td>
<td>height</td>
<td>(i)</td>
<td>0.037641</td>
</tr>
<tr>
<td>ME</td>
<td>height</td>
<td>(ii)</td>
<td>0.039139</td>
</tr>
<tr>
<td>ME</td>
<td>area</td>
<td>(iii)</td>
<td>0.145236</td>
</tr>
<tr>
<td>MO/ME</td>
<td>height</td>
<td>(i)</td>
<td>0.038948</td>
</tr>
<tr>
<td>MO/ME</td>
<td>height</td>
<td>(ii)</td>
<td>0.036997</td>
</tr>
<tr>
<td>MO/ME</td>
<td>area</td>
<td>(iii)</td>
<td>0.140535</td>
</tr>
</tbody>
</table>
Table 3.4.5.4.2: IR determined % trans content established using peak heights and various sets of calibration data (straight baseline with wavenumber parameters: N1 = 995 cm\(^{-1}\), N2 = 969 cm\(^{-1}\), N3 = 944 cm\(^{-1}\)).

<table>
<thead>
<tr>
<th>Sample</th>
<th>MS/ME</th>
<th>ME</th>
<th>MO/ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT001a</td>
<td>50.3</td>
<td>49.4</td>
<td>51.9</td>
</tr>
<tr>
<td>FT001b</td>
<td>49.8</td>
<td>48.7</td>
<td>51.4</td>
</tr>
<tr>
<td>FT001c</td>
<td>50.1</td>
<td>49.0</td>
<td>51.7</td>
</tr>
<tr>
<td>FT001d</td>
<td>51.2</td>
<td>50.1</td>
<td>52.8</td>
</tr>
<tr>
<td>FT001e</td>
<td>49.8</td>
<td>48.7</td>
<td>51.4</td>
</tr>
<tr>
<td><strong>mean</strong></td>
<td><strong>50.2</strong></td>
<td><strong>49.2</strong></td>
<td><strong>51.8</strong></td>
</tr>
<tr>
<td><strong>std. dev.</strong></td>
<td><strong>0.6</strong></td>
<td><strong>0.6</strong></td>
<td><strong>0.6</strong></td>
</tr>
<tr>
<td><strong>C.V.</strong></td>
<td><strong>1.1%</strong></td>
<td><strong>1.2%</strong></td>
<td><strong>1.1%</strong></td>
</tr>
<tr>
<td><strong>Diff.</strong></td>
<td><strong>10.4%</strong></td>
<td><strong>8.1%</strong></td>
<td><strong>13.9%</strong></td>
</tr>
<tr>
<td>FT002a</td>
<td>30.4</td>
<td>28.0</td>
<td>31.4</td>
</tr>
<tr>
<td>FT002b</td>
<td>30.5</td>
<td>28.0</td>
<td>31.4</td>
</tr>
<tr>
<td>FT002c</td>
<td>30.3</td>
<td>27.8</td>
<td>31.2</td>
</tr>
<tr>
<td>FT002d</td>
<td>30.5</td>
<td>28.0</td>
<td>31.4</td>
</tr>
<tr>
<td>FT002e</td>
<td>30.7</td>
<td>28.3</td>
<td>31.7</td>
</tr>
<tr>
<td><strong>mean</strong></td>
<td><strong>30.5</strong></td>
<td><strong>28.0</strong></td>
<td><strong>31.4</strong></td>
</tr>
<tr>
<td><strong>std. dev.</strong></td>
<td><strong>0.2</strong></td>
<td><strong>0.2</strong></td>
<td><strong>0.2</strong></td>
</tr>
<tr>
<td><strong>C.V.</strong></td>
<td><strong>0.6%</strong></td>
<td><strong>0.7%</strong></td>
<td><strong>0.6%</strong></td>
</tr>
<tr>
<td><strong>Diff.</strong></td>
<td><strong>1.8%</strong></td>
<td><strong>-6.5%</strong></td>
<td><strong>4.8%</strong></td>
</tr>
<tr>
<td>FT003a</td>
<td>2.7</td>
<td>-1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>FT003b</td>
<td>2.7</td>
<td>-1.7</td>
<td>2.7</td>
</tr>
<tr>
<td>FT003c</td>
<td>2.9</td>
<td>-1.5</td>
<td>2.9</td>
</tr>
<tr>
<td>FT003d</td>
<td>2.8</td>
<td>-1.6</td>
<td>2.8</td>
</tr>
<tr>
<td>FT003e</td>
<td>2.9</td>
<td>-1.6</td>
<td>2.8</td>
</tr>
<tr>
<td><strong>mean</strong></td>
<td><strong>2.8</strong></td>
<td><strong>-1.6</strong></td>
<td><strong>2.8</strong></td>
</tr>
<tr>
<td><strong>std. dev.</strong></td>
<td><strong>0.1</strong></td>
<td><strong>0.1</strong></td>
<td><strong>0.1</strong></td>
</tr>
<tr>
<td><strong>C.V.</strong></td>
<td><strong>3.2%</strong></td>
<td><strong>5.6%</strong></td>
<td><strong>3.3%</strong></td>
</tr>
<tr>
<td><strong>Diff.</strong></td>
<td><strong>-0.4%</strong></td>
<td><strong>-158%</strong></td>
<td><strong>-1.8%</strong></td>
</tr>
<tr>
<td>FT004a</td>
<td>1.8</td>
<td>-2.7</td>
<td>1.7</td>
</tr>
<tr>
<td>FT004b</td>
<td>1.6</td>
<td>-2.9</td>
<td>1.6</td>
</tr>
<tr>
<td>FT004c</td>
<td>1.7</td>
<td>-2.8</td>
<td>1.6</td>
</tr>
<tr>
<td>FT004d</td>
<td>1.8</td>
<td>-2.6</td>
<td>1.7</td>
</tr>
<tr>
<td>FT004e</td>
<td>1.5</td>
<td>-2.7</td>
<td>1.4</td>
</tr>
<tr>
<td><strong>mean</strong></td>
<td><strong>1.7</strong></td>
<td><strong>-2.7</strong></td>
<td><strong>1.6</strong></td>
</tr>
<tr>
<td><strong>std. dev.</strong></td>
<td><strong>0.2</strong></td>
<td><strong>0.2</strong></td>
<td><strong>0.2</strong></td>
</tr>
<tr>
<td><strong>C.V.</strong></td>
<td><strong>8.9%</strong></td>
<td><strong>3.0%</strong></td>
<td><strong>9.3%</strong></td>
</tr>
<tr>
<td><strong>Diff.</strong></td>
<td><strong>23.5%</strong></td>
<td><strong>-300%</strong></td>
<td><strong>17.6%</strong></td>
</tr>
</tbody>
</table>

Note: the diff. values quoted in the tables represents the % difference between the corresponding IR (a) value and the GC estimated IR (b) value i.e. \(\frac{(a - b)}{b} \times 100\).
Table 3.4.5.4.3; IR determined % trans content found using peak height and various sets of calibration data heights (straight baseline with wavenumber parameters: \( N_1 = 985 \text{ cm}^{-1}, N_2 = 969 \text{ cm}^{-1}, N_3 = 952 \text{ cm}^{-1} \)).

<table>
<thead>
<tr>
<th>Sample</th>
<th>MS/ME</th>
<th>ME</th>
<th>MO/ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT001a</td>
<td>49.6</td>
<td>46.2</td>
<td>51.5</td>
</tr>
<tr>
<td>FT001b</td>
<td>49.1</td>
<td>46.6</td>
<td>51.0</td>
</tr>
<tr>
<td>FT001c</td>
<td>49.7</td>
<td>46.9</td>
<td>51.3</td>
</tr>
<tr>
<td>FT001d</td>
<td>50.5</td>
<td>46.9</td>
<td>52.4</td>
</tr>
<tr>
<td>FT001e</td>
<td>49.1</td>
<td>45.5</td>
<td>51.0</td>
</tr>
<tr>
<td>mean</td>
<td>49.7</td>
<td>46.0</td>
<td>51.5</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.6</td>
<td>0.6</td>
<td>0.6</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.2%</td>
<td>1.2%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Diff.</td>
<td>8.8%</td>
<td>1.1%</td>
<td>13.1%</td>
</tr>
<tr>
<td>FT002a</td>
<td>29.9</td>
<td>26.8</td>
<td>31.1</td>
</tr>
<tr>
<td>FT002b</td>
<td>29.9</td>
<td>26.8</td>
<td>31.2</td>
</tr>
<tr>
<td>FT002c</td>
<td>29.8</td>
<td>26.7</td>
<td>31.1</td>
</tr>
<tr>
<td>FT002d</td>
<td>29.9</td>
<td>26.8</td>
<td>31.2</td>
</tr>
<tr>
<td>FT002e</td>
<td>30.3</td>
<td>27.2</td>
<td>31.5</td>
</tr>
<tr>
<td>mean</td>
<td>29.9</td>
<td>26.8</td>
<td>31.2</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>C.V.</td>
<td>0.6%</td>
<td>0.7%</td>
<td>0.6%</td>
</tr>
<tr>
<td>Diff.</td>
<td>0.03%</td>
<td>-10.3%</td>
<td>4.2%</td>
</tr>
<tr>
<td>FT003a</td>
<td>2.0</td>
<td>-0.5</td>
<td>2.2</td>
</tr>
<tr>
<td>FT003b</td>
<td>1.9</td>
<td>-0.6</td>
<td>2.2</td>
</tr>
<tr>
<td>FT003c</td>
<td>2.1</td>
<td>-0.4</td>
<td>2.4</td>
</tr>
<tr>
<td>FT003d</td>
<td>2.0</td>
<td>-0.5</td>
<td>2.3</td>
</tr>
<tr>
<td>FT003e</td>
<td>2.1</td>
<td>-0.4</td>
<td>2.4</td>
</tr>
<tr>
<td>mean</td>
<td>2.0</td>
<td>-0.5</td>
<td>2.3</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>C.V.</td>
<td>4.6%</td>
<td>-17.6%</td>
<td>4.1%</td>
</tr>
<tr>
<td>Diff.</td>
<td>-28.6%</td>
<td>-117.1%</td>
<td>-18.2%</td>
</tr>
<tr>
<td>FT004a</td>
<td>1.4</td>
<td>-1.1</td>
<td>1.7</td>
</tr>
<tr>
<td>FT004b</td>
<td>1.2</td>
<td>-1.3</td>
<td>1.5</td>
</tr>
<tr>
<td>FT004c</td>
<td>1.3</td>
<td>-1.2</td>
<td>1.6</td>
</tr>
<tr>
<td>FT004d</td>
<td>1.5</td>
<td>-1.0</td>
<td>1.7</td>
</tr>
<tr>
<td>FT004e</td>
<td>1.2</td>
<td>-1.1</td>
<td>1.4</td>
</tr>
<tr>
<td>mean</td>
<td>1.3</td>
<td>-1.1</td>
<td>1.6</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>C.V.</td>
<td>9.8%</td>
<td>-9.7%</td>
<td>8.6%</td>
</tr>
<tr>
<td>Diff.</td>
<td>-3.7%</td>
<td>-182.4%</td>
<td>15.4%</td>
</tr>
</tbody>
</table>

Note: the diff. values quoted in the tables represents the % difference between the corresponding IR (a) value and the GC estimated IR (b) value (i.e. = \(((a - b)/b)*100\)).
Table 3.4.5.4.4: IR determined % trans content using peak heights and MS/ME calibration data (straight baseline with wavenumber parameters: \( N1 = \text{varied}, N2 = 969 \text{ cm}^{-1}, N3 = \text{varied} \)).

<table>
<thead>
<tr>
<th>Sample</th>
<th>% trans content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MS/ME</td>
</tr>
<tr>
<td>FT001a</td>
<td>51.3</td>
</tr>
<tr>
<td>FT001b</td>
<td>50.6</td>
</tr>
<tr>
<td>FT001c</td>
<td>51.0</td>
</tr>
<tr>
<td>FT001d</td>
<td>52.1</td>
</tr>
<tr>
<td>FT001e</td>
<td>50.6</td>
</tr>
<tr>
<td>mean</td>
<td>51.1</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.6</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.2%</td>
</tr>
<tr>
<td>Diff.</td>
<td>12.4%</td>
</tr>
<tr>
<td>FT002a</td>
<td>30.2</td>
</tr>
<tr>
<td>FT002b</td>
<td>30.2</td>
</tr>
<tr>
<td>FT002c</td>
<td>30.0</td>
</tr>
<tr>
<td>FT002d</td>
<td>30.2</td>
</tr>
<tr>
<td>FT002e</td>
<td>30.5</td>
</tr>
<tr>
<td>mean</td>
<td>30.2</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.2</td>
</tr>
<tr>
<td>C.V.</td>
<td>0.6%</td>
</tr>
<tr>
<td>Diff.</td>
<td>0.9%</td>
</tr>
<tr>
<td>FT003a</td>
<td>2.5</td>
</tr>
<tr>
<td>FT003b</td>
<td>2.3</td>
</tr>
<tr>
<td>FT003c</td>
<td>2.4</td>
</tr>
<tr>
<td>FT003d</td>
<td>2.4</td>
</tr>
<tr>
<td>FT003e</td>
<td>2.5</td>
</tr>
<tr>
<td>mean</td>
<td>2.4</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.1</td>
</tr>
<tr>
<td>C.V.</td>
<td>2.7%</td>
</tr>
<tr>
<td>Diff.</td>
<td>-13.9%</td>
</tr>
<tr>
<td>FT004a</td>
<td>1.8</td>
</tr>
<tr>
<td>FT004b</td>
<td>1.7</td>
</tr>
<tr>
<td>FT004c</td>
<td>1.7</td>
</tr>
<tr>
<td>FT004d</td>
<td>1.9</td>
</tr>
<tr>
<td>FT004e</td>
<td>1.6</td>
</tr>
<tr>
<td>mean</td>
<td>1.8</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.1</td>
</tr>
<tr>
<td>C.V.</td>
<td>6.4%</td>
</tr>
<tr>
<td>Diff.</td>
<td>28.7%</td>
</tr>
</tbody>
</table>

Note: the diff. values quoted in the tables represents the % difference between the corresponding IR (a) value and the GC estimated IR (b) value (i.e. \( = \frac{(a - b)b}{100} \)).
Table 3.4.5.4.5: IR determined % trans content found using peak areas and various sets of calibration data (used program AREA3.0y and wavenumber parameters:- N1 = 976 cm\(^{-1}\), N2 = 964 cm\(^{-1}\)).

<table>
<thead>
<tr>
<th>Sample</th>
<th>MS/ME</th>
<th>ME</th>
<th>MO/ME</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT001a</td>
<td>49.7</td>
<td>48.9</td>
<td>51.0</td>
</tr>
<tr>
<td>FT001b</td>
<td>49.0</td>
<td>48.2</td>
<td>50.3</td>
</tr>
<tr>
<td>FT001c</td>
<td>49.3</td>
<td>48.5</td>
<td>50.6</td>
</tr>
<tr>
<td>FT001d</td>
<td>50.67</td>
<td>49.9</td>
<td>52.1</td>
</tr>
<tr>
<td>FT001e</td>
<td>48.7</td>
<td>47.8</td>
<td>50.0</td>
</tr>
<tr>
<td>mean</td>
<td>49.5</td>
<td>48.64</td>
<td>50.8</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.8</td>
<td>0.78</td>
<td>0.8</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.6%</td>
<td>1.8%</td>
<td>1.6%</td>
</tr>
<tr>
<td>Diff.</td>
<td>8.7%</td>
<td>6.9%</td>
<td>11.6%</td>
</tr>
</tbody>
</table>

| FT002a | 29.8   | 29.0  | 30.5  |
| FT002b | 9.7    | 28.9  | 30.4  |
| FT002c | 29.5   | 28.8  | 30.3  |
| FT002d | 29.9   | 29.1  | 30.6  |
| FT002e | 30.2   | 29.5  | 31.0  |
| mean   | 29.8   | 29.1  | 30.5  |
| std. dev. | 0.3   | 0.3   | 0.3   |
| C.V.   | 0.9%   | 0.9%  | 0.9%  |
| Diff.  | -0.4%  | -2.9% | 2.0%  |

| FT003a | 1.7    | 1.0   | 1.5   |
| FT003b | 1.6    | 0.9   | 1.4   |
| FT003c | 1.7    | 1.1   | 1.6   |
| FT003d | 1.7    | 1.0   | 1.5   |
| FT003e | 1.7    | 1.0   | 1.6   |
| mean   | 1.7    | 1.0   | 1.5   |
| std. dev. | 0.1   | 0.1   | 0.1   |
| C.V.   | 4.0%   | 6.5%  | 4.5%  |
| Diff.  | 40.4%  | -64.3%| 45.0% |

| FT004a | 1.3    | 0.7   | 1.2   |
| FT004b | 1.2    | 0.5   | 1.0   |
| FT004c | 1.2    | 0.5   | 1.0   |
| FT004d | 1.4    | 0.7   | 1.2   |
| FT004e | 1.2    | 0.5   | 1.0   |
| mean   | 1.2    | 0.6   | 1.1   |
| std. dev. | 0.1   | 0.1   | 0.1   |
| C.V.   | 8.6%   | 17.7% | 9.6%  |
| Diff.  | -9.6%  | -58.1%| -19.1%|

Note; the diff. values quoted in the tables represents the % difference between the corresponding IR (a) value and the GC estimated IR (b) value (i.e. = ((a - b)/b)*100)).
Table 3.4.5.4.6: IR determined % trans content found using peak areas and MS/ME calibration data (used program AREA2.oxy and wavenumber parameters: N1 varied, N2 = 976 cm⁻¹, N3 = 964 cm⁻¹, N4 varied).

<table>
<thead>
<tr>
<th>Sample</th>
<th>% trans content</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT001a</td>
<td>51.8</td>
</tr>
<tr>
<td>FT001b</td>
<td>51.2</td>
</tr>
<tr>
<td>FT001c</td>
<td>51.7</td>
</tr>
<tr>
<td>FT001d</td>
<td>52.9</td>
</tr>
<tr>
<td>FT001e</td>
<td>51.3</td>
</tr>
<tr>
<td>Mean</td>
<td>51.8</td>
</tr>
<tr>
<td>Std. dev.</td>
<td>0.7</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.3%</td>
</tr>
<tr>
<td>Diff.</td>
<td>13.9%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>% trans content</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT002a</td>
<td>30.7</td>
</tr>
<tr>
<td>FT002b</td>
<td>30.6</td>
</tr>
<tr>
<td>FT002c</td>
<td>30.4</td>
</tr>
<tr>
<td>FT002d</td>
<td>30.6</td>
</tr>
<tr>
<td>FT002e</td>
<td>31.0</td>
</tr>
<tr>
<td>Mean</td>
<td>30.7</td>
</tr>
<tr>
<td>Std. dev.</td>
<td>0.2</td>
</tr>
<tr>
<td>C.V.</td>
<td>0.6</td>
</tr>
<tr>
<td>Diff.</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>% trans content</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT003a</td>
<td>2.7</td>
</tr>
<tr>
<td>FT003b</td>
<td>2.5</td>
</tr>
<tr>
<td>FT003c</td>
<td>2.6</td>
</tr>
<tr>
<td>FT003d</td>
<td>2.6</td>
</tr>
<tr>
<td>FT003e</td>
<td>2.7</td>
</tr>
<tr>
<td>Mean</td>
<td>2.6</td>
</tr>
<tr>
<td>Std. dev.</td>
<td>0.1</td>
</tr>
<tr>
<td>C.V.</td>
<td>2.5%</td>
</tr>
<tr>
<td>Diff.</td>
<td>6.1%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>% trans content</th>
</tr>
</thead>
<tbody>
<tr>
<td>FT004a</td>
<td>2.0</td>
</tr>
<tr>
<td>FT004b</td>
<td>1.9</td>
</tr>
<tr>
<td>FT004c</td>
<td>1.9</td>
</tr>
<tr>
<td>FT004d</td>
<td>2.1</td>
</tr>
<tr>
<td>FT004e</td>
<td>1.8</td>
</tr>
<tr>
<td>Mean</td>
<td>1.9</td>
</tr>
<tr>
<td>Std. dev.</td>
<td>0.1</td>
</tr>
<tr>
<td>C.V.</td>
<td>5.6%</td>
</tr>
<tr>
<td>Diff.</td>
<td>42.8%</td>
</tr>
</tbody>
</table>

Note: the diff. values quoted in the tables represents the % difference between the corresponding IR (a) value and the GC estimated IR (b) value [i.e. = ((a - b)/b)*100]].
Table 3.4.5.4.7; Composition of trial fats determined by capillary GC. Data provided by MAFF.

<table>
<thead>
<tr>
<th>FAME</th>
<th>% OF FAME</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FT001a</td>
</tr>
<tr>
<td>8:0</td>
<td>0.0</td>
</tr>
<tr>
<td>10:0</td>
<td>0.0</td>
</tr>
<tr>
<td>12:0</td>
<td>0.11</td>
</tr>
<tr>
<td>14:0</td>
<td>0.15</td>
</tr>
<tr>
<td>15:0</td>
<td>0.0</td>
</tr>
<tr>
<td>16:0</td>
<td>7.74</td>
</tr>
<tr>
<td>16:1t</td>
<td>0.15</td>
</tr>
<tr>
<td>16:1c</td>
<td>0.10</td>
</tr>
<tr>
<td>17:0</td>
<td>0.06</td>
</tr>
<tr>
<td>18:0</td>
<td>9.62</td>
</tr>
<tr>
<td>18:1t</td>
<td>37.10</td>
</tr>
<tr>
<td>18:1c</td>
<td>27.50</td>
</tr>
<tr>
<td>18:2tt</td>
<td>0.79</td>
</tr>
<tr>
<td>18:2ct</td>
<td>0.53</td>
</tr>
<tr>
<td>18:2cc</td>
<td>0.39</td>
</tr>
<tr>
<td>18:3ccc</td>
<td>0.49</td>
</tr>
<tr>
<td>19:0</td>
<td>0.15</td>
</tr>
<tr>
<td>20:0</td>
<td>0.86</td>
</tr>
<tr>
<td>20:1t</td>
<td>0.73</td>
</tr>
<tr>
<td>20:1c</td>
<td>0.59</td>
</tr>
<tr>
<td>22:0</td>
<td>0.73</td>
</tr>
<tr>
<td>22:1t</td>
<td>0.19</td>
</tr>
<tr>
<td>22:1c</td>
<td>0.16</td>
</tr>
<tr>
<td>24:0</td>
<td>0.0</td>
</tr>
<tr>
<td>unident.</td>
<td>11.8</td>
</tr>
<tr>
<td>mono-c</td>
<td>28.4</td>
</tr>
<tr>
<td>mono-t</td>
<td>38.2</td>
</tr>
<tr>
<td>sat.</td>
<td>19.3</td>
</tr>
<tr>
<td>polysat.</td>
<td>2.4</td>
</tr>
<tr>
<td>total-t</td>
<td>39.9</td>
</tr>
<tr>
<td>total-c</td>
<td>29.9</td>
</tr>
<tr>
<td>IR-t</td>
<td>40.7</td>
</tr>
<tr>
<td>IR-c</td>
<td>30.4</td>
</tr>
</tbody>
</table>

Notes: unident. = unidentified FAME's; mono-c = cis mono-unsaturated; mono-t = trans mono-unsaturated; sat. = saturated; total-t = total trans content; total-c = total cis content; IR-t = GC estimated IR trans content (see Table 3.4.5.3.3); IR-C = GC estimated IR cis content.

FT001b was reassayed by the MAFF after comparison of the results of FT001a with the corresponding IR results.
Before the results are interpreted, a short explanation will be given concerning the rationale for the use of the new wavenumber parameters and area measurements applied in this section. For peak area measurements, it is recognised, that this can offer improved accuracy and precision over peak height measurements. In the case of the variable wavenumber parameters, this technique was used in an attempt to correct for the trans band broadening with increase of percentage trans content and degree of overlap of the interfering bands, as illustrated by figures 3.4.4.1 and 3.4.4.2. Finally, the area absorbance measurements using the straight baseline parameters \((N1 = 976 \text{ cm}^{-1}, N2 = 964 \text{ cm}^{-1})\) were used because these measured the top of the trans band, avoiding the contributions from the overlapping bands.

Examination of the composition of trial fats, determined by capillary GC (Table 3.4.5.4.7), shows that their trans content varied from 1.4% to 45.0%. The same table also shows that approximately 5% of the FAME’s are unidentified for each fat, which means that the true percentage trans value of the fats are not known, and that the accuracy of the various IR techniques cannot be established. However, the repeatability of the IR results was established. No chromatograms were provided by the MAFF for these fats, so the influence of peak overlap upon the results could not be assessed. Table 3.4.5.4.1 shows the regression data from the various calibration plots used to establish the trans content. All the plots showed excellent linearity over the trans range studied. In the case of the ME/MS calibration data, the two highest trans content solutions had absorbance values which were greater than 2.0 and hence not included in the generation of the calibration plot data shown in Table 3.4.5.4.1.

In order to simplify the task of interpreting these results, comparisons of IR results with GC estimated IR values will be made separately for each fat, followed by a general conclusion.

\textbf{FT001}: All the IR methods produced higher values than the GC estimated IR value (FT001a - 45.5% trans content). IR results generated using the ME calibration data and the peak height parameters (ii) were significantly lower than the other IR methods, which is in accordance with the conclusion reached in the previous section. All the other methods gave values that were in the range 48.6% to 51.8%. Those results generated using the MO/ME calibration data always gave slightly higher trans values than the MS/ME calibration data using the corresponding baseline parameters. MS/ME calibration data using the variable baseline parameters generated % trans results which were significantly higher than those generated using MS/ME and the fixed baseline parameters, when either peak heights or areas were utilised. The peak height data had a lower coefficient of variance (1.2%) than the peak area data (approx. 1.6%). According to these
IR results, most of the unidentified FAME's in the GC chromatogram are trans.

FT002; The mean results generated using the ME calibration date and baseline parameters (i) and (ii) were 28% and 26.9%, respectively, which were both lower than the GC estimated IR value (29.9%). Results from all the other IR methods, including those generated using the ME with area parameters (iii), were very similar to the GC estimated IR results. Furthermore, identical trends for the trans results were observed for the calibration solutions and baseline parameters to those described for FT001. GC results show that approximately 5% of the FAME's were unidentified. IR results suggest that only a small percentage of these FAME's are trans. The coefficients of variance for the IR results were lower than those obtained for FT001 for both the peak height and peak area results, however, the peak area results were slightly higher.

FT003 and FT004; Similar trends were observed for these fats, which have low levels of trans content according to GC (GC estimated IR trans contents of 2.8% and 1.4%, respectively) and approximately 5% unidentified FAME's. Once again, those results generated using the ME calibration data, parameters (i) and (ii), were significantly lower than either the GC estimated IR or other IR methods. Surprisingly, the results generated with MO/ME calibration data, which are well outside the calibration range of these samples, were very similar to the MS/ME results using the corresponding baseline parameters (see Tables 3.4.5.4.2, 3.4.5.4.3 and 3.4.5.4.5.). However, there were appreciable differences in results for different baseline parameters. Clearly, the selection of the baseline has a considerable influence upon the trans results. Those results generated using the baseline parameter set (i) on Table 3.4.5.4.2 showed the smallest difference between the MS/ME and MO/ME calibration results, and also between these values and the GC estimated IR values.

In the case of the peak area results, obtained with fixed baseline parameters (iii) in Table 3.4.5.4.5, the two-component solution results were considerably lower than those using the other baseline parameters for peak height or area. On the other hand, the reverse was true for ME. Examination of the solute calibration spectra of methyl elaidate with the baseline corresponding to this parameter set revealed that the line drawn between the points is almost parallel to the x-axis for all the spectra, as demonstrated by figure 3.4.5.4.1. This was not the case for the two-component calibration solution which showed that the angle of the line drawn between the two points changed with trans content (see figures 3.4.5.4.2). Therefore, this set of baseline parameters are affected by the absorbance of the interfering bands. The MS/ME results using the peak height and area with variable baseline parameters, in Tables 3.4.5.4.4 and
3.4.5.4.6, were both slightly lower than the GC estimated IR value for fat FT003, and both higher for fat FT004. These differences may be attributed to the different parameters used in the peak area and height determination. Again, it is clear that the main problem is the selection of the baseline. The same conclusion has been reached by other authors who have recommended that baselines of appropriate calibration spectra be superimposed on the sample spectra.

In conclusion, the GC method was able to establish the composition of the fats, which the IR methods could not, but the total trans content by GC was uncertain due to the inability of the method to identify and resolve all the FAME's. IR results gave a single value, which was the total trans content of the fat. Repeatability experiments for the fats with high trans content (i.e. FT001 and FT002) gave low coefficients of variation which were slightly higher for the peak area than the peak height results. Coefficient of variance results for the fats with low trans levels (FT003 and FT004) were considerably higher, but this should be interpreted with caution, because the lower the trans value the greater the error expected, which is also true for GC peaks. It was interesting to note that the standard deviations of the various IR methods for the respective fats were very similar, despite the different coefficients of variance.

Comparison of the IR results confirm that both of the two-component calibration solutions compensate for the interfering bands which overlap with the trans bands, whereas the methyl elaidate solutions do not. These results also show that small differences are observed when methyl oleate or methyl stearate are used with methyl elaidate in the two-component set of calibration solutions. Unfortunately, no conclusions can be reached regarding this issue for the results of the fats with low trans levels, where the differences would be expected to be enhanced, because these fats were outside the calibration range of the MO/ME calibration data. However, very similar results were obtained using MS/ME and MO/ME calibration data for these fats. Re-examination of the literature showed that previous workers have used two other FAME's, in conjunction with methyl elaidate, to produce sets of two-component calibration solutions; methyl oleate (Lancer et al., 1988) and methyl linoleate (Sleeter et al., 1989; Madison, 1982). Further studies are therefore recommended to establish the effects of using either different chainlength saturated, or cis poly-unsaturated FAME's as the second component in the two-component calibration solutions.

Significant differences were observed in the trans content results generated using the same set of calibration spectra but different baseline wavenumber parameters. These differences are enhanced in the fats with low trans content, and demonstrate that the fixed wavenumber parameters do not compensate correctly for the interfering bands which are prominent when the
trans content is low. Two alternative baseline parameters were tried, the first measured the peak area of the top of the trans peak, effectively ignoring the base where the overlap with the interfering bands occurs. This baseline technique was found to be affected by the interfering bands and did not overcome the problem. The second used wavenumber parameters which were varied to adjust for the trans band broadening with trans content. Peak height and area measurements were used and produced slightly different results, but these were attributed to differences in wavenumber parameters. The main problem encountered with this approach was the selection of the variable baseline parameters. Other problems may be encountered if the profile of the interfering bands in the calibration spectra (i.e. methyl oleate or methyl stearate) does not match that of the sample.

As the true trans content values of these fats are not known, it can not be said which of the above IR methods gives the most accurate result. However, the experiments do show the necessity for a new standard IR method which uses a two-component set of calibration solutions. The selection of the baseline parameters and compositions of the standards requires careful specification.

3.4.6 Cis -C-H stretching band quantitative studies

3.4.6.1 Extinction coefficients of the cis band at $\sim 3005 \text{ cm}^{-1}$

of various FAME's

The infrared absorption spectra of cis FAME's show a band at $\sim 3005 \text{ cm}^{-1}$ on the shoulder of the methylene (-CH$_2$-) stretching band. This band is attributed to the stretching vibration of the cis C=C-H carbon hydrogen bond. The measurement of the absorbance of this band under controlled conditions is the basis of the IR techniques used to determine the cis content of fats and oils.

An IR spectroscopic method of determining the cis content of FAME's based on the absorbance of the $3005 \text{ cm}^{-1}$ band was investigated. Studies were made of this band for various cis FAME's. Trans and saturated FAME's were also studied in order to establish whether they possess bands which may interfere. The method used the spectra of a set of calibration solutions (see section
3.3.1.a) taken using the standard scanning conditions (50 co-added scans, 1 mm pathlength cell), from which the solvent component was subtracted using a normalisation factor of 1. The straight baseline correction technique described in section 3.3.4 was used to establish the absorbance of the band, incorporating the baseline parameters indicated in Table 3.4.6.1. Plots of the peak absorbance (baseline corrected) versus concentration of the corresponding FAME were produced. The extinction coefficients were obtained by dividing the gradient of the plot by the cell thickness in cm. Plots were forced through the origin.

The plots of the cis FAME's showed excellent adherence to Beer's law over the concentration range studied. Comparison of the extinction coefficient ($k_1$, in terms of moles/litre), determined from the plots, showed similar values for the mono-ene cis FAME's. Cis FAME's with two and three isolated double bonds had $k_1$ values which were twice and three times, respectively, the value of the mono-enes. This clearly indicates that the $k_1$ of the cis band at $\approx 3005$ cm$^{-1}$ is proportional to the number of isolated cis double bonds present. Differences may be attributed to the error in the baseline correction technique used to eliminate the overlapping bands. On the other hand, $k_2$ (in terms of grams/litre) values show a different trend which is the same as that found for the $k_2$ values for the trans band in section 3.4.3. An increase in the number of cis double bonds is accompanied by an increase in the wavenumber of the peak ($N_2$ shows slight shift to higher wavenumber).

Two unsaturated alcohols {(OH)18:1$\Delta$9c and (OH)18:3$\Delta$9c$\Delta$12c15c} were also studied in order to establish whether the carbonyl group, which is absent in these compounds, influences the cis =C-H stretching band. Results obtained in Table 3.4.6.1 indicate that there is no interaction. Studies on saturated and trans FAME's revealed the presence of two bands at $\approx 3022$ cm$^{-1}$ and 2990 cm$^{-1}$, which can potentially cause problems in the baseline correction due to overlap. These bands were found to be of greater intensity in trans FAME's than saturated FAME's (see figure 3.4.6.1.1 and Table 3.4.6.1), furthermore, these bands increased in intensity with increase in the degree of trans unsaturation. These results support the work in section 2.4.2.3, in which studies on methyl-d$_3$-elaidate suggest that two weak bands attributed to the trans ethylenic bond absorb in the same regions as the C-H stretching modes of the carbomethoxy group.
Table 3.4.6.1: Shows the extinction coefficient of the cis =C-H stretching band for various cis FAME's (calculated from the calibration plots). The k of a band, likely to interfere, present in saturated and trans FAME's is also shown.

<table>
<thead>
<tr>
<th>FAME</th>
<th>Wavenumber parameters used in baseline correction</th>
<th>Extinction coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(N_1)</td>
<td>(N_2)</td>
</tr>
<tr>
<td>18:1Δ9c</td>
<td>3036</td>
<td>3005</td>
</tr>
<tr>
<td>(OH)18:1Δ9c</td>
<td>3034</td>
<td>3006</td>
</tr>
<tr>
<td>20:1Δ11c</td>
<td>3036</td>
<td>3005</td>
</tr>
<tr>
<td>22:1Δ13c</td>
<td>3036</td>
<td>3005</td>
</tr>
<tr>
<td>24:1Δ15c</td>
<td>3036</td>
<td>3005</td>
</tr>
<tr>
<td>18:2Δ9c12c</td>
<td>3042</td>
<td>3011</td>
</tr>
<tr>
<td>18:3Δ9c12c15c</td>
<td>3043</td>
<td>3013</td>
</tr>
<tr>
<td>(OH)18:3Δ9c12c15c</td>
<td>3042</td>
<td>3013</td>
</tr>
<tr>
<td>20:3Δ11c14c17c</td>
<td>3034</td>
<td>3013</td>
</tr>
<tr>
<td>16:1Δ9t</td>
<td>3046</td>
<td>3021</td>
</tr>
<tr>
<td>16:1Δ6t</td>
<td>3046</td>
<td>3021</td>
</tr>
<tr>
<td>18:1Δ9t</td>
<td>3046</td>
<td>3022</td>
</tr>
<tr>
<td>18:1Δ9t(dg)</td>
<td>3046</td>
<td>3028</td>
</tr>
<tr>
<td>18:1Δ9t12(OH)</td>
<td>3046</td>
<td>3022</td>
</tr>
<tr>
<td>18:1Δ11t</td>
<td>3046</td>
<td>3020</td>
</tr>
<tr>
<td>20:1Δ11t</td>
<td>3045</td>
<td>3021</td>
</tr>
<tr>
<td>22:1Δ13t</td>
<td>3046</td>
<td>3021</td>
</tr>
<tr>
<td>18:2Δ9t12t</td>
<td>3048</td>
<td>3025</td>
</tr>
<tr>
<td>20:2Δ11t14t</td>
<td>3048</td>
<td>3025</td>
</tr>
<tr>
<td>18:0</td>
<td>3040</td>
<td>3028</td>
</tr>
</tbody>
</table>

Note: \((\text{OH})18:1\Delta 9c = \text{oetyl alcohol and (OH)18:3\Delta 9c12c18c = \text{linolenyl alcohol}}.\)
To conclude, the $k_2$ values in Table 3.4.6.1 of cis FAME's suggest that errors will occur if this band is used to calculate the cis content as a percentage weight of methyl oleate, when large quantities of cis poly-unsaturated or cis mono-enes of fatty acid chainlength other than C18 are present. Furthermore, bands present in saturated or trans FAME's may cause errors in the determination of the cis band absorbance when the straight baseline correction technique is used. The influence of these interfering bands is described in section 3.4.6.2.

3.4.6.2 Determination of the cis contents of simulated mixtures using the absorbance of the cis =C-H band at 3005 cm$^{-1}$

According to results obtained in the previous section, saturated and trans FAME's possess bands at $\approx$ 3022 cm$^{-1}$ and 2990 cm$^{-1}$ which lie on either side of the cis =C-H stretching band (3005 cm$^{-1}$). Simulated mixtures of methyl oleate/methyl stearate (MO/MS) and methyl oleate/methyl elaidate (MO/ME) were prepared to establish whether these bands interfere with the straight baseline correction technique.

The spectra of the simulated mixtures and methyl oleate calibration solutions were produced using 1 mm and 2 mm liquid cells. Identical baseline wavenumber parameters were used to obtain the cis peak height absorbance values of all the simulated and calibration spectra. These were as follows:

$$N_1 = 3036 \text{ cm}^{-1}, \quad N_2 = \text{peak maximum (3005 cm}^{-1}), \quad N_3 = 2986 \text{ cm}^{-1}$$

Tables 3.4.6.2.1 and 3.4.6.2.2 show the percentage cis content of the standard mixtures of methyl oleate/methyl elaidate and methyl oleate/methyl stearate, determined by the IR method described above. These results show that the difference between the known and determined cis content increases with decrease in the cis content of the mixture. The reasons for the differences are:

a. The band at 3022 cm$^{-1}$, present in both the methyl stearate and methyl elaidate spectra, causes an error in the baseline.

b. The use of a straight baseline correction technique to establish the absorbance of a band which lies on the shoulder of a stronger band.
The effects of these faults are illustrated in figure 3.4.6.2.1, which shows the resultant solute spectrum of a standard mixture of methyl oleate/methyl stearate in the region 3100 to 2980 cm⁻¹.

Table 3.4.6.2.1; Cis content of methyl oleate/methyl elaidate determined using the cis =C-H stretching band absorbance. Values in brackets are differences between determined and known cis values.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>% cis content (w/w)</th>
<th>1 mm cell</th>
<th>2 mm cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Known</td>
<td>determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>38</td>
<td>25.4 (-12.6)</td>
<td>25.6 (-12.4)</td>
</tr>
<tr>
<td>(b)</td>
<td>71</td>
<td>64.4 (-6.6)</td>
<td>64.7 (-6.3)</td>
</tr>
<tr>
<td>(c)</td>
<td>83</td>
<td>81.3 (-1.7)</td>
<td>81.4 (-1.6)</td>
</tr>
<tr>
<td>(d)</td>
<td>90.8</td>
<td>90 (-0.8)</td>
<td>90.5 (-0.3)</td>
</tr>
<tr>
<td>(e)</td>
<td>97.5</td>
<td>97.3 (-0.2)</td>
<td>94.8 (-2.7)</td>
</tr>
</tbody>
</table>

Table 3.4.6.2.2; Cis content of methyl oleate/methyl stearate determined using the cis =C-H stretching band absorbance. Values in brackets are differences between determined and known cis values.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>% cis content (w/w)</th>
<th>1 mm cell</th>
<th>2 mm cell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Known</td>
<td>determined</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(a)</td>
<td>60.1</td>
<td>54.9 (-5.2)</td>
<td>54.8 (-5.3)</td>
</tr>
<tr>
<td>(b)</td>
<td>40.1</td>
<td>33.6 (-6.5)</td>
<td>32.9 (-7.2)</td>
</tr>
<tr>
<td>(c)</td>
<td>30.1</td>
<td>22.6 (-7.5)</td>
<td>21.7 (-8.4)</td>
</tr>
<tr>
<td>(d)</td>
<td>20</td>
<td>11.9 (-8.1)</td>
<td>13.7 (-6.3)</td>
</tr>
<tr>
<td>(e)</td>
<td>10</td>
<td>0.9 (-9.1)</td>
<td>2.2 (-7.8)</td>
</tr>
<tr>
<td>(f)</td>
<td>4</td>
<td>-5.6 (-9.6)</td>
<td>-5.9 (-9.9)</td>
</tr>
<tr>
<td>(g)</td>
<td>2</td>
<td>-7.7 (-9.7)</td>
<td>-8.3 (-10.3)</td>
</tr>
</tbody>
</table>
3.4.6.3 Determination of the cis content of fats and oils using the cis \( \equiv \text{C-H} \) stretching band absorbance

A final evaluation of the IR method described in the previous section was carried out on a selection of real samples. The following fats and oils were assayed by the IR and capillary GC methods:

**FATS**

FT001, FT002, FT003, FT004: see section 3.4.5.4 and Table 3.4.5.4.7 for the GC compositions.

**OILS**

Sunflower and gamma oil purchased from a local store

These fats and oils were chosen because they cover a wide range of fatty acid composition. The GC results for fats FT001 and FT002 (see Table 3.4.5.4.7) show that these samples contain high levels of trans and saturated FAME's and as predicted from the results of the previous section, the IR % cis content results (see table 3.4.6.3.2) were considerably lower than the GC values. In the case of fats FT003 and FT004 the levels of trans and saturated FAME's were considerably lower than the previous samples (between 10 and 20%), however, the IR % cis contents were still substantially lower than the GC values (see table 3.4.6.3.2).

In the case of sunflower and gamma oil, no trans and low levels (less than 8%) of saturated FAME's were found to be present by the GC method (see Table 3.4.4.3.1). Therefore the problems in the IR method described for the previous samples will not occur with these oils. Cis content values of 168% and 173% were obtained for sunflower and gamma oil, respectively, by the IR method. These results are close to the GC estimated IR values (see Table 3.4.4.3.2). This emphasises the other problem with the IR method, namely, that cis di-unsaturated and tri-unsaturated FAME's have extinction coefficients for the cis \( \equiv \text{C-H} \) band which are twice and three times, respectively, that of the cis mono-unsaturated FAME's. Thus, the IR technique measures the total number of isolated cis double bonds, which is useful if one wishes to determine the iodine number, but produces significant error if one wishes to determine the cis content when there is a high percentage of poly-unsaturated FAME's.
Table 3.4.6.3.1; Composition of oils determined by capillary GC (mean values from two chromatograms shown).

<table>
<thead>
<tr>
<th>FAME</th>
<th>% CONTENT</th>
<th>Sunflower oil</th>
<th>Gamma oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>0.0</td>
<td>0.0</td>
<td>0.46</td>
</tr>
<tr>
<td>14:0</td>
<td>0.01</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>16:0</td>
<td>4.96</td>
<td>4.55</td>
<td></td>
</tr>
<tr>
<td>16:1c</td>
<td>0.02</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>18:0</td>
<td>1.47</td>
<td>0.39</td>
<td></td>
</tr>
<tr>
<td>18:1c</td>
<td>17.26</td>
<td>4.83</td>
<td></td>
</tr>
<tr>
<td>18:2cc</td>
<td>76.17</td>
<td>84.47</td>
<td></td>
</tr>
<tr>
<td>18:3ccc</td>
<td>0.01</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>18:3ccc*</td>
<td>0.0</td>
<td>5.27</td>
<td></td>
</tr>
<tr>
<td>20:3ccc</td>
<td>0.01</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>22:0</td>
<td>0.09</td>
<td>0.0</td>
<td></td>
</tr>
<tr>
<td>total cis</td>
<td>93.47</td>
<td>94.59</td>
<td></td>
</tr>
<tr>
<td>sat.</td>
<td>6.53</td>
<td>5.41</td>
<td></td>
</tr>
<tr>
<td>IR est.</td>
<td>169.68</td>
<td>189.64</td>
<td></td>
</tr>
</tbody>
</table>

Note: sat. = total % content of saturated FAME's, total cis = total % content of cis FAME's, IR est. = GC estimated IR % content (used following equation = % mono-cis + 2 x % di-cis + 3 x tri-cis), 18:3ccc* represents gamma methyl linolenate.

Table 3.4.6.3.2; % cis content of various fats and oils determined by the IR (=C-H band) and capillary GC method.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% cis content</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GC estimated IR</td>
</tr>
<tr>
<td>FT001</td>
<td>32.4</td>
</tr>
<tr>
<td>FT002</td>
<td>34.1</td>
</tr>
<tr>
<td>FT003</td>
<td>110.7</td>
</tr>
<tr>
<td>FT004</td>
<td>107.2</td>
</tr>
<tr>
<td>Sunflower oil</td>
<td>169.7</td>
</tr>
<tr>
<td>Gamma oil</td>
<td>189.6</td>
</tr>
</tbody>
</table>

Note the GC established IR results were determined by substituting the corresponding GC results into the equation quoted on Table 3.4.6.3.1. The following set of wavenumber parameters define the straight baseline used throughout (N1 = 3036 cm⁻¹, N2 = peak maximum (value in last column), N3 = 2986 cm⁻¹).
Further work on this band was discontinued because of the inherent problems outlined above. The author believes that this band may be used to determine the cis content of samples, but only when a suitable de-convolution technique is made available that can resolve the bands in this region, thus removing the need for any baseline correction. Resolution might also be possible between the cis =C-H stretching bands of mono-, di- and tri-unsaturated FAME's because their peak maxima are located at different frequencies.

3.4.7 Cis C=C stretching bands quantitative studies

3.4.7.1 Extinction coefficient of the cis and trans C=C stretching band of various FAME's using the straight baseline correction technique

In section 2.4.3, the C=C stretching bands of cis and trans FAME's in the liquid phase spectra were discussed. These bands have a very similar appearance in the solution spectra. They occur on the shoulder of the carbonyl band at approximately 1654 cm\(^{-1}\) and 1668 cm\(^{-1}\) for the cis and trans FAME's respectively, and their intensities are very low (see figure 3.4.7.1.1). The performance of most dispersive IR spectrometers is inadequate for the quantitative exploitation of these bands. FTIR spectroscopy offers the ability of studying low intensity bands by accumulating spectra to reduce noise level. Accordingly, FTIR techniques were applied to establish the feasibility of using the bands for quantitative analysis.

The main difficulty was establishing the correct baseline. The straight baseline technique (section 3.3.4) was tried first, and the following conditions were used:

- Pathlength of cell = 1 mm
- Resolution = 2 cm\(^{-1}\)
- Scans accumulated = 50
- Subtraction of solvent component: normalisation factor = 1

Plots of corrected peak absorbance versus concentration (moles/litre) of the FAME's were produced. Extinction coefficients were determined by dividing the slope of the plot (forced through the origin) by the pathlength. The plots for the C=C stretching band of cis FAME's
obeyed Beer's law well. The corresponding band of trans FAME's is much weaker and absorbance measurements were only possible on the most concentrated solutions. Thus the method is not suitable for trans FAME's.

Table 3.4.7.1.1 shows the extinction coefficient of the cis C=C band of several FAME's. Similar values were obtained for methyl oleate and methyl erucate, whereas the value for methyl linolenate (which contains three isolated cis double bonds) was approximately two and a half times that shown by the cis mono-enes. Close examination of the C=C stretching region of methyl linolenate revealed that the cis peak was broader than the corresponding band of methyl oleate, furthermore a slight inflection was observed. Similar observations were evident in the spectra of other non-conjugated poly-unsaturated FAME's, and appear to be caused by interactions of the double bonds (see section 2.4.3).

Table 3.4.7.1.1; C=C stretching band extinction coefficient (k) of various FAME's obtained using the straight baseline technique. Note (a): k could not be obtained due to the high noise level.

<table>
<thead>
<tr>
<th>FAME</th>
<th>BASELINE PARAMETERS (cm⁻¹)</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N1</td>
<td>peak position</td>
</tr>
<tr>
<td>18:1Δ9c</td>
<td>1664</td>
<td>1654</td>
</tr>
<tr>
<td>22:1Δ13c</td>
<td>1664</td>
<td>1654</td>
</tr>
<tr>
<td>18:3Δ9c12c15c</td>
<td>1670</td>
<td>1653</td>
</tr>
<tr>
<td>mono-trans</td>
<td>-</td>
<td>1668</td>
</tr>
<tr>
<td>FAME's</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3.4.7.2 Cis content determination of model methyl oleate/methyl elaidate mixtures using the straight baseline correction technique

The preliminary results in the previous section suggest that the cis C=C band could be used quantitatively. To establish the feasibility of the quantitative use of the cis band, several mixtures of methyl elaidate and methyl oleate were prepared. The cis content of each mixture was
determined by measuring the cis C=C band absorbance using the straight baseline correction technique. Spectra used were produced with a 1 mm cell and the previously stated conditions. Equation quoted in section 3.3.13 was used in conjunction with the k value of methyl oleate produced using the same pathlength cell and conditions.

The results in Table 3.4.7.2.1 indicate that the cis contents determined by IR were approximately 3% high for mixtures (b) to (e), but 5% low for mixture (a). The low absorbance of the cis peak and high noise levels in the resultant solute spectra were the likely causes of the discrepancies.

Table 3.4.7.2.1; Determination of the cis content of methyl oleate and methyl elaidate mixtures using the cis C=C stretching band and the straight baseline technique (1 mm pathlength cell).

<table>
<thead>
<tr>
<th>mixture</th>
<th>Known cis content of mixture</th>
<th>IR determined cis content of mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>38</td>
<td>33 (-5)</td>
</tr>
<tr>
<td>(b)</td>
<td>71</td>
<td>72.2 (+1.2)</td>
</tr>
<tr>
<td>(c)</td>
<td>83</td>
<td>86.4 (+3.4)</td>
</tr>
<tr>
<td>(d)</td>
<td>90.8</td>
<td>94 (+3.2)</td>
</tr>
<tr>
<td>(e)</td>
<td>97.5</td>
<td>100.4 (+2.9)</td>
</tr>
</tbody>
</table>

Note: values in brackets = difference between the known and determined values.

Solutions to this problem include: (i) increasing the pathlength of the liquid cell, (ii) increasing the number of scans accumulated per spectrum. The latter option was not considered suitable because 50 scans were accumulated to produce these spectra, and a considerable increase in this number would be required to obtain any significant reduction in the noise. Therefore the only option open was to use a longer pathlength cell.
Table 3.4.7.2.2; Cis content of methyl elaidate and methyl oleate standard mixtures by the IR technique (using a 2 mm cell) and GC methods. The values in brackets are differences between the known and determined values.

<table>
<thead>
<tr>
<th>Mixture</th>
<th>Known (wt/wt)</th>
<th>cis content (% wt/wt)</th>
<th>IR (2 mm)</th>
<th>G.C.</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a)</td>
<td>38</td>
<td>28.3 (-9.7)</td>
<td>38.9 (0.9)</td>
<td></td>
</tr>
<tr>
<td>(b)</td>
<td>71</td>
<td>65.7 (-5.3)</td>
<td>70.6 (-0.4)</td>
<td></td>
</tr>
<tr>
<td>(c)</td>
<td>83.1</td>
<td>81.8 (-1.3)</td>
<td>82.7 (-0.4)</td>
<td></td>
</tr>
<tr>
<td>(d)</td>
<td>90.8</td>
<td>90.3 (-0.5)</td>
<td>90.2 (-0.6)</td>
<td></td>
</tr>
<tr>
<td>(e)</td>
<td>97.5</td>
<td>96.3 (-1.2)</td>
<td>97 (-0.5)</td>
<td></td>
</tr>
</tbody>
</table>

The spectra of the standard mixtures of methyl oleate and methyl elaidate were retaken using a 2 mm pathlength cell. Figure 3.4.7.2.1 show the solute spectra of the standard mixtures (a), (b) and (e), produced with the 1 mm and 2 mm cells. These spectra clearly show that increasing the pathlength successfully eliminates the noise problem. Unexpectedly, the results obtained with the 2 mm cell (Table 3.4.7.2.2) showed greater differences from the known values when the trans content exceeded 10%. This was attributed, surprisingly, to the improved quality of the spectra produced with the 2 mm cell, in which absorbance of the trans band at 1668 cm\(^{-1}\) produced an error in the baseline absorbance value as illustrated by figure 3.4.7.2.2. The net effect was a reduction in the cis peak absorbance value and thus a low cis content. This error increased with increase in trans content. In the case of the 1 mm cell spectra, the trans peak was obscured by the noise, which in turn lowered the absorbance of the baseline, thus increasing the cis C=C band absorbance value (see figure 3.4.7.2.1).

The GC method produced values within 1% of the known values of the mixtures, throughout the concentration range studied. A number of smaller peaks were observed in the GC chromatogram of the mixtures. These were impurities accounting for 1% to 2% of the total area. It should be noted that no corrections were made for these impurities in either the GC or IR method results.

The following experiment was conducted in order to establish whether the problems incurred in
the use of the straight baseline technique also occurred when the trans C=C band is absent. In this experiment the cis C=C band was used to determine the cis content of several standard mixtures of methyl oleate and methyl stearate varying in cis content from 2% to 60% (wt/wt). Two different pathlength cells (1 mm and 2 mm) were used to take the solution spectra of the mixtures, methyl oleate standards and solvent using the scanning conditions and subtraction process stipulated in the previous section. The following wavenumber parameters were used to determine the cis peak absorbance of both the standard and mixtures:

\[ N1 = 1670 \text{ cm}^{-1} \quad N2 = 1654 \text{ cm}^{-1} \quad N3 = 1630 \text{ cm}^{-1} \]

Table 3.4.7.2.3; Cis content determined by the IR technique using the cis C=C band and the GC technique. The numbers in brackets are the differences between the known and determined values.

<table>
<thead>
<tr>
<th>mixture</th>
<th>cis content % (wt/wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>known</td>
</tr>
<tr>
<td>a.</td>
<td>60.1</td>
</tr>
<tr>
<td>b.</td>
<td>40.1</td>
</tr>
<tr>
<td>c.</td>
<td>30.1</td>
</tr>
<tr>
<td>d.</td>
<td>20.0</td>
</tr>
<tr>
<td>e.</td>
<td>10.0</td>
</tr>
<tr>
<td>f.</td>
<td>4.0</td>
</tr>
<tr>
<td>g.</td>
<td>2.0</td>
</tr>
</tbody>
</table>

Plots of the cis peak absorbance vs. concentration of the methyl oleate standards produced using the 1 mm and 2 mm cell showed excellent adherence to Beer's law. The extinction coefficients obtained from these plots were used to determine the cis content of the mixtures (corresponding pathlength). Once again the cis content was also determined using the GC technique for comparison.
Table 3.4.7.2.3 shows the percentage cis content of each standard mixture determined by the IR method using a 1 mm and 2 mm pathlength cell. Also included on this table are the GC results. In general the differences between the known and IR determined cis content were found to increase with decrease in cis content. Once again, the results obtained by the GC method were within 1% of the known values in all cases regardless of concentration.

Figure 3.4.7.2.3 shows the resultant solute spectrum of methyl stearate. Clearly no bands were observed in this region which suggested that the problems encountered with the mixtures of methyl oleate and methyl elaidate should not occur, due to the absence of the trans C=C band. This however was not the case, which implies that further error or errors are present in the IR method.

The cause of the error was identified to be the use of the straight baseline technique for a band on a shoulder of the much larger band. This is best illustrated by the spectra of mixture (f) (see figure 3.4.7.2.4), where the peak absorbance value (baseline corrected) is negative due to the very low absorbance of the cis peak and curvature of the shoulder.

3.4.7.3 Cis C=C band extinction coefficient of cis poly-unsaturated FAME’s using the straight baseline correction technique

Two straight baseline correction parameters were used to determine the extinction coefficients of the two overlapping cis C=C stretching bands present in methyl linoleate (see Table 3.4.7.3.1). Using this technique, the band at the lower wavenumber, referred to as band b, had an extinction coefficient one and a half times that of methyl oleate. Similarly, methyl linolenate, containing three isolated cis double bonds, was found to have an unsymmetrical C=C band with an extinction coefficient two and a half times that of methyl oleate (see Table 3.4.7.3.1). This suggests that in mixtures high in poly-unsaturated FAME’s, the calculated cis content will be high when determined using the straight baseline correction technique, but low if the total number of isolated cis double bonds are calculated. Although the results were rather negative, the characteristic differences observed between cis poly-unsaturated FAME’s were considered sufficient to allow determination of cis FAME’s varying in degree of unsaturation.
Table 3.4.7.3.1: Cis C=C extinction coefficients (k) of various poly-unsaturated FAME's established using a 2 mm cell and the straight baseline correction technique.

<table>
<thead>
<tr>
<th>FAME</th>
<th>wavenumber parameters used</th>
<th>k</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N1</td>
<td>N2</td>
</tr>
<tr>
<td>18:2Δ9c12c</td>
<td>1672</td>
<td>1656</td>
</tr>
<tr>
<td>band a</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:2Δ9c12c</td>
<td>1672</td>
<td>1648</td>
</tr>
<tr>
<td>band b</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:3Δ9c12c15c</td>
<td>1670</td>
<td>1653</td>
</tr>
</tbody>
</table>

3.4.7.4 Development of a curved baseline technique in the determination of the peak height of the cis C=C band

The preliminary quantitative studies on the cis C=C band were unsuccessful due to the inability of the straight baseline correction technique to compensate for the absorbance of the overlapping carbonyl band. Two approaches to a solution of the problem were tried:

1. The use of a simulated curved baseline generated by a computer program.

2. The use of the spectrum of methyl stearate, which contains no C=C stretching bands, to simulate the curved baseline.

Attempts to use a cubic spline program in the first approach were unsuccessful in simulating the curvature of the carbonyl shoulder. Before the second approach could be tried, it was necessary to establish whether or not the profile of the carbonyl shoulder was the same for all FAME's in the region of the cis band.

Initial studies on the spectra of methyl stearate, of varying concentrations (six solutions in the range 13.4x10^-3 to 40x10^-3 moles/litre, taken using a 1 mm and 2 mm cell, making a total of 12 spectra), revealed that when the spectra were overlayed, after solvent component subtraction and normalisation, the profile of the carbonyl shoulder changed (see figure 3.4.7.4.1). This
change was attributed to solute-solute interaction, and also observed in the spectra of other FAME's (see figure 3.4.7.4.2).

To overcome this problem, an obey program called SIM4.oy was developed which selects the best fitting spectrum of methyl stearate, from a library of twelve spectra, to simulate the profile of the carbonyl band with which the cis or trans C=C stretching bands overlap (see figure 3.4.7.4.3). After selection, the program then subtracts the methyl stearate spectrum from the sample spectrum, before calculating either the cis or trans C=C band absorbance. Further details of this technique, which will be referred to as the curved baseline simulation (CBS) technique, can be found in section 3.3.5.

Surprisingly good subtraction of the carbonyl shoulder was obtained using this new technique. This is best illustrated by figure 3.4.7.4.4, which shows the spectra of methyl oleate, methyl elaidate and the corresponding cis and trans C=C bands after subtraction of the carbonyl shoulder.

3.4.7.5  Cis C=C stretching band extinction coefficient of various FAME's using the cis curved baseline simulation technique

The extinction coefficients of the cis C=C stretching band of various FAME's determined using the new CBS technique are shown in Table 3.4.7.5.1. All the FAME's plots of cis C=C band absorbance versus concentration (example; 13x10^-3 M to 135x10^-3 M), used to obtain the extinction coefficient, showed excellent adherence to Beer's law. Thus indicating that the new technique was successful at eliminating the interfering carbonyl band.

Very few studies have been conducted on the intensity or position of the C=C stretching vibrations in the IR. However, the intensity of this band is expected to diminish when it is moved from the end of a chain towards the centre, because the molecule becomes more symmetrical. This has been demonstrated by Kletz et al. (1948), who drew attention to the fall in intensity of this band in a series of trimethylpentenes in which the symmetry was gradually increased. No studies have been conducted on the effects of symmetry on the intensity of the double bond stretching vibration in cis and trans (non-centrosymmetric) isomers.
The results obtained in Table 3.4.7.5.1 show that little or no change was observed in the intensity of the cis C=C stretch when methylene groups (-CH₂-) are added or removed from either side chain X or Y, thus increasing or decreasing the symmetry. The only exceptions were 18:1Δ11c, 18:1Δ9c(OH) and oleyl alcohol. Differences associated with 18:1Δ11c may be attributed to the disparity of the methylene chains on either side of the double bond and hence a reduction in symmetry, resulting in a larger C=C band intensity. However, several of the FAME's studied possess greater differences between the methylene chains, but were found to have k values close to the to methyl oleate value. Further investigations are required to establish the cause of this anomaly.

In the case of methyl ricinoleate (18:1Δ9c12(OH)), the intensity of the C=C stretching vibration was expected to be higher than that of the corresponding mono-unsaturated FAME's, due to the presence of the alcohol group on the twelfth carbon, which significantly reduces the symmetry of the molecule. However, the band was found to be of lower intensity. This suggests that the hydroxyl group is interacting with the cis C=C stretching vibration, possibly due to hydrogen bonding between the alcohol hydrogen and the C=C double bond, as demonstrated by figure 3.4.7.5.1. Hydrogen bonding of this type has been suggested to occur in o-propenylphenol and o-allylphenol by Varsanyi et al. (1969).

Oleyl alcohol was found to have an C=C band intensity equal to that of methyl ricinoleate, which is lower than the respective band in mono-unsaturated FAME's. The alternative baseline correction technique (straight baseline) was used to calculate the absorbance of oleyl alcohol, due to the absence of the carbonyl band. However, this was not considered to be the reason for the lower than expected intensity. This reduction in intensity may again be due to hydrogen bonding between the double bond and the hydrogen on the alcohol group as suggested for methyl ricinoleate. Bonding of this type may be intermolecular or intramolecular due to the flexibility of the molecule.
Table 3.4.7.5.1: Cis C=C stretching band extinction coefficients (k) of various mono-unsaturated FAME’s, determined using the new CBS.

<table>
<thead>
<tr>
<th>FAME</th>
<th>No. of methylenes</th>
<th>cell</th>
<th>k</th>
<th>S(M) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>M</td>
<td>(mm)</td>
<td></td>
</tr>
<tr>
<td>14:1Δ9c</td>
<td>7</td>
<td>3</td>
<td>2</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>18:1Δ9c</td>
<td>7</td>
<td>7</td>
<td>1a</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>1a</td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td></td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td></td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>(OH)18:1Δ9c</td>
<td>7</td>
<td>7</td>
<td>2b</td>
<td>5.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>2.5b</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18:1Δ9c12(OH))</td>
<td>7</td>
<td>5</td>
<td>2a</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td>2a</td>
<td></td>
<td></td>
<td>1.2</td>
</tr>
<tr>
<td>18:1A11c</td>
<td>9</td>
<td>5</td>
<td>2</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>19:1A10c</td>
<td>8</td>
<td>7</td>
<td>2.5</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.8</td>
</tr>
<tr>
<td>20:1Δ8c</td>
<td>6</td>
<td>10</td>
<td>1a</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.9</td>
</tr>
<tr>
<td></td>
<td>2.5a</td>
<td></td>
<td></td>
<td>1.3</td>
</tr>
<tr>
<td></td>
<td>2.5a</td>
<td></td>
<td></td>
<td>1.1</td>
</tr>
<tr>
<td>20:1Δ11c</td>
<td>9</td>
<td>7</td>
<td>2</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.6</td>
</tr>
<tr>
<td>20:1Δ13c</td>
<td>11</td>
<td>5</td>
<td>1a</td>
<td>6.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>2.5a</td>
<td></td>
<td></td>
<td>2.2</td>
</tr>
<tr>
<td>22:1Δ13c</td>
<td>11</td>
<td>7</td>
<td>1</td>
<td>6.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.0</td>
</tr>
</tbody>
</table>

Note, M and N represent the number of methylenes on either side of the double bond (CH₃(CH₂)ⁿ-CH=CH-(CH₂)ₘ-COOCH₃). A represents different calibration spectra taken on separate days. S(M) = standard deviation of points from line in the calibration plot obtained using the linear regression traversing the origin. (OH)18:1Δ9c = oleyl alcohol (b = straight baseline was used because carbonyl band is not present). Baseline parameters used: N1 = 1676 cm⁻¹, N2 = 1654 cm⁻¹, N3 = 1630 cm⁻¹.

147
Determination of the cis content of model mixtures using the cis C=C band and the CBS technique

After the successful determination of the C=C band absorbance of various cis mono-unsaturated FAME's using the CBS procedure, which simulated and subtracted the overlapping carbonyl shoulder, the same technique was used to determine the cis content of methyl oleate and methyl stearate mixtures. Spectra were taken using standard scanning conditions (50 scans co-added) and two different pathlength liquid cells (1 mm and 2 mm). A normalisation factor of one was used to subtract the solvent component from each spectrum before the CBS technique was applied. Determination of the cis content was achieved using the equation in section 3.3.13 and calibration data generated from methyl oleate standards (obtained in the same manner using the same pathlength cells).

Table 3.4.7.6.1 shows the percentage cis content of each standard mixture of methyl oleate/methyl stearate, determine by the CBS IR technique (using 1 mm and 2 mm cells) and the capillary GC technique. The results obtained using the IR technique were generally slightly lower than the known values, whereas, the reverse was true of the GC technique. Increasing the pathlength from 1 mm to 2 mm had the nett effect of reducing the difference between the known and determined values.

<table>
<thead>
<tr>
<th>sol.</th>
<th>cis content (% wt./wt.)</th>
<th>conc. (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>known</td>
<td>IR (1 mm cell)</td>
</tr>
<tr>
<td>(a)</td>
<td>60</td>
<td>57.8 (-1.2)</td>
</tr>
<tr>
<td>(b)</td>
<td>40</td>
<td>38.3T (-1.7)</td>
</tr>
<tr>
<td>(c)</td>
<td>30</td>
<td>28.6 (-1.4)</td>
</tr>
<tr>
<td>(d)</td>
<td>20</td>
<td>19.0 (-1.0)</td>
</tr>
<tr>
<td>(e)</td>
<td>10</td>
<td>8.7 (-1.3)</td>
</tr>
<tr>
<td>(f)</td>
<td>4</td>
<td>3.2 (-0.8)</td>
</tr>
<tr>
<td>(g)</td>
<td>2</td>
<td>0.9 (-1.1)</td>
</tr>
</tbody>
</table>

Note. T = poor baseline fit, * = noisy baseline spectrum used responsible for large difference.
Figures 3.4.7.6.1 and 3.4.7.6.2 show the baseline spectra superimposed on the spectra of the mixtures. All of the baseline spectra fitted the carbonyl band remarkably well, except for solutions (e) and (g) (taken with the 2 mm cell), where the baseline spectra were noisy, due to over multiplication. Thus introducing an error into the cis peak absorbance value. Taking this into account, the IR result obtained using the 2 mm cell are comparable to the corresponding GC results.

After such encouraging preliminary results, mixtures of methyl elaidate and methyl oleate were assayed in the same manner, to establish whether the corresponding trans C=C stretching band overlaps to any extent and therefore introduces an error. Results obtained are shown in Table 3.4.7.6.2. The results obtained with the 2 mm cell were significantly better than those obtained using the 1 mm cell, as expected. However, the differences between the known and IR values were considerable greater than those observed in the previous experiment. Furthermore, the difference was found to increase with increase in trans content.

Table 3.4.7.6.3; Cis content of standard mixtures (methyl oleate/methyl elaidate; total concentration of FAME's kept constant for each mixture) determined by the new IR (CBS) technique using a 2 mm cell.

<table>
<thead>
<tr>
<th>sol.</th>
<th>cis content (% wt./wt.)</th>
<th>conc. (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>known</td>
<td>determined IR (2 mm cell)</td>
</tr>
<tr>
<td>(a)</td>
<td>39</td>
<td>41.3 (2.3)</td>
</tr>
<tr>
<td>(b)</td>
<td>59</td>
<td>60.1 (1.1)</td>
</tr>
<tr>
<td>(c)</td>
<td>79.3</td>
<td>81.6 (2.3)</td>
</tr>
<tr>
<td>(d)</td>
<td>89.6</td>
<td>91.3 (1.7)</td>
</tr>
<tr>
<td>(e)</td>
<td>95.8</td>
<td>97.6 (1.8)</td>
</tr>
<tr>
<td>(f)</td>
<td>97.9</td>
<td>98.9 (1.0)</td>
</tr>
</tbody>
</table>
Examination of figure 2.4.3.10, which shows the superimposed spectra of the cis and trans C=C stretching bands (after subtraction of the carbonyl band), clearly shows that the contribution from the trans band, to the cis peak absorbance is small. It is therefore difficult to attribute all of the difference between the known and established values to band overlap. The only other variable was the concentration of sample; as this decreased the error increased.

To verify the effect of concentration, an additional set of methyl oleate/methyl elaidate mixtures was assayed, in which the total concentration of FAME's was kept constant. The results (see Table 3.4.7.6.3) show a significant decrease in the difference between the determined and known cis content (all of the differences were smaller than 2.5%). These results suggest that the error is mainly attributed to the quantity of sample present. For example the level of error has an inverse relationship with concentration.

Although the main error in the method can be attributed to concentration effects, the absorbance contribution of the trans C=C overlap should not be overlooked. From the results obtained in the last experiment, the cis content results can be expected to be at least 1% higher than the known value when the trans content exceeds 50% (wt./wt.) of the sample.

### Table 3.4.7.6.2: Cis content of standard mixtures (methyl oleate/methyl elaidate; varying in total concentration of FAME's) determined by capillary GC and the new IR (curved baseline simulation) technique.

<table>
<thead>
<tr>
<th>sol.</th>
<th>cis content (% wt./wt.)</th>
<th>conc. (g/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>known</td>
<td>determined</td>
</tr>
<tr>
<td></td>
<td></td>
<td>IR (1 mm cell)</td>
</tr>
<tr>
<td>(a)</td>
<td>38</td>
<td>45.4 (7.4)</td>
</tr>
<tr>
<td>(b)</td>
<td>71</td>
<td>77.1 (6.1)</td>
</tr>
<tr>
<td>(c)</td>
<td>83.1</td>
<td>89.2 (6.1)</td>
</tr>
<tr>
<td>(d)</td>
<td>90.7</td>
<td>95.0 (4.3)</td>
</tr>
<tr>
<td>(e)</td>
<td>97.5</td>
<td>98.3 (0.8)</td>
</tr>
</tbody>
</table>
3.4.7.7 Extinction coefficient of the cis C=C stretching band of poly-unsaturated FAME's

Poly-unsaturated FAME's with isolated cis double bonds possess C=C stretching bands in the IR. Certain correlations have been established between the number of bands and the number of double bonds. This has been discussed in detail in section 2.4.3. Unfortunately, the cis C=C stretching bands of poly-unsaturated FAME's overlap with the corresponding band in cis mono-unsaturated FAME's. This clearly introduces an error into any cis content determination of fats and oils high in cis poly-unsaturated FAME's.

In order to assess the degree of error introduced by different poly-unsaturated FAME's, their cis C=C band extinction coefficient were established. Various different cells were used and in general the best results (e.g. spectra with best baseline fits and least noise) were obtained using the longest pathlength (2.5 mm). This was also found to be the optimum pathlength, as longer pathlengths reduce light through-put, and thus increase noise. Furthermore, the C=C band is no longer visible on account of the strongly absorbing carbonyl and solvent bands.

Table 3.4.7.7.1; Cis C=C stretching band extinction coefficients (k) of various cis isolated poly-unsaturated FAME's, determined using the new CBS technique.

<table>
<thead>
<tr>
<th>FAME</th>
<th>cell (mm)</th>
<th>Peak maxima (cm⁻¹)</th>
<th>Extinction coefficient</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2Δ9c12c</td>
<td>1</td>
<td>band a. 1654</td>
<td>9.5 (0.9%)</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>band b. 1647</td>
<td>10.3 (1.4%)</td>
<td>1.5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>band a. 1654</td>
<td>9.5 (0.4%)</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>band b. 1654</td>
<td>9.8 (0.4%)</td>
<td>1.5</td>
</tr>
<tr>
<td>18:3Δ6c9c12c</td>
<td>1</td>
<td>1653</td>
<td>14.4 (1.5%)</td>
<td>2.2</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1653</td>
<td>16.3 (0.8%)</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1653</td>
<td>15.3 (0.8%)</td>
<td>2.3</td>
</tr>
<tr>
<td>20:3Δ11c14c17c</td>
<td>1</td>
<td>1653</td>
<td>16.1 (0.4%)</td>
<td>2.4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1653</td>
<td>16.9 (0.7%)</td>
<td>2.5</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1653</td>
<td>16.2 (0.6%)</td>
<td>2.4</td>
</tr>
<tr>
<td>20:3Δ6c11c14c</td>
<td>1</td>
<td>1653</td>
<td>15.1 (1.1%)</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>1653</td>
<td>16.2 (4.1%)</td>
<td>2.4</td>
</tr>
</tbody>
</table>

151
Abbreviations used in Table 3.4.7.7.1; cell = pathlength of cell (mm). Values in brackets represent standard deviation of points in calibration plot (conc. [g/l] vs. absorbance) from regression line traversing the origin. Ratio = FAME cis band k / methyl oleate k.

Table 3.4.7.7.1 shows the cis C=C bands k values of various poly-unsaturated FAME's. The "ratio" values shown in Table 3.4.7.7.1 are the k value of the respective FAME divided by the k value of methyl oleate. This value can be used to estimate at what percentage, with respect to methyl oleate, each poly-unsaturated FAME's has to be present in a mixture to produce errors of say, 1% or 10% in the cis content using the IR technique. Simple algebra was used as shown in the example below;

**EXAMPLE**

Objective; to determine the percentage level (with respect to methyl oleate) that methyl linoleate must be present in a mixture containing methyl oleate to produce an error of 1%.

The following assumptions are made;

\[ x + y = 100 \]

This equation states that the % of methyl oleate plus methyl linoleate equals the total cis content (100%), because it is assumed that none of the other FAME's present possess cis bonds.

\[ x + yf = 101 \]

This equation takes into account the different intensities of the cis C=C band in methyl oleate and methyl linoleate. An error of 1% is assumed, hence 101 on the right hand side.

Where;

- \( x \) = % methyl oleate
- \( y \) = % methyl linoleate
- \( f \) = k ratioed value of methyl linoleate (e.g. cis C=C band k value of methyl linoleate divided by the corresponding k value of methyl oleate.)

Taking ratio = 1.5 as an example
By solving these equations simultaneously, $x = 98\%$ and $y = 2\%$. Thus, 2% methyl linoleate has to be present with respect to the combined cis-FAME concentration to produce an error of 1% in the cis content. Table 3.4.7.7.2 summarises the levels of each cis poly-unsaturated FAME required to produce an error of 1% and 10% in the cis content.

**Table 3.4.7.7.2;** Percentage of cis poly-unsaturated FAME, relative to methyl oleate concentration, which will give an error of 1% and 10% in the cis content value determined for a mixture using the cis C=C band CBS IR technique. The ratio values were selected from Table 3.4.7.7.1.

<table>
<thead>
<tr>
<th>FAME</th>
<th>Ratio value used</th>
<th>Percentage of PUF</th>
<th>Error of 1%</th>
<th>Error of 10%</th>
</tr>
</thead>
<tbody>
<tr>
<td>18:2Δ9c12c</td>
<td>1.5</td>
<td></td>
<td>2.0</td>
<td>20</td>
</tr>
<tr>
<td>18:3Δ6c9c12c</td>
<td>2.4</td>
<td></td>
<td>0.4</td>
<td>4.2</td>
</tr>
<tr>
<td>20:3Δ11c14c17c</td>
<td>2.5</td>
<td></td>
<td>0.4</td>
<td>4.0</td>
</tr>
<tr>
<td>20:3Δ8c11c14c</td>
<td>2.4</td>
<td></td>
<td>0.4</td>
<td>4.0</td>
</tr>
</tbody>
</table>
The spectra of the following methyl and ethyl sorbate isomers were taken, in order to investigate the main factors influencing the frequency of C=C stretching bands of conjugated isomers.

Methyl trans trans sorbate

Methyl cis trans sorbate

Ethyl trans trans sorbate
Table 3.4.7.8.1; Positions of C=O and C=C stretching bands of various isomers of methyl and ethyl sorbate. Where; L = liquid film, H = melt, c and t = cis and trans respectively.

<table>
<thead>
<tr>
<th>Isomer</th>
<th>C=O stretching</th>
<th>C=C stretching</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>band (cm⁻¹)</td>
<td>bands (cm⁻¹)</td>
</tr>
<tr>
<td>ME-CH=CH-CH=CH-CO₂-ME</td>
<td>1721</td>
<td>1646</td>
</tr>
<tr>
<td>t t ME-CH=CH-CH=CH-CO₂·ME H</td>
<td>1718</td>
<td>1642</td>
</tr>
<tr>
<td>t t ME-CH=CH-CH=CH-CO₂·ET L</td>
<td>1715</td>
<td>1647</td>
</tr>
</tbody>
</table>

Table 3.4.7.8.1 shows the positions of the C=O and C=C stretching bands of two isomers of methyl sorbate and one of ethyl sorbate. The following correlations deduced from these results were supported by data from Allen et al. (1955) shown in the next table. The spectra of the compounds in these tables all showed the expected number of C=C bands, two for the methyl sorbate isomers, and one for the others. The conversion of either the 2, 3 or the 4, 5 - ethylenic bond from trans to cis causes a shift of the band to lower frequency. Thus, the trans trans isomer absorbs at 1642 and 1614 cm⁻¹, and the cis cis isomer at 1623 and 1587 cm⁻¹, whilst the mixed isomers show intermediate values.

When the results in Tables 3.4.7.8.1 and 3.4.7.8.2 are compared with those in section 2.4.3, in which conjugated isomer mixtures prepared from methyl linolate were studied (see Table 3.4.7.8.3, and figure 2.4.3.11), certain correlations in band positions are observed. The intensities of the C=C bands in the isomer mixtures are considerably lower than those of the methyl sorbate isomers. This decrease in the intensities is because the C=C groups are not conjugation to the carbonyl group.

According to the above results the trans, trans and cis, trans (or trans, cis) conjugated isomers of methyl linolate posses C=C bands which overlap with the cis isolated C=C stretching band. The presence of these conjugated isomers is therefore expected to introduce an error in the cis % content determined by the IR technique.
Table 3.4.7.8.2; Results taken from paper by Allen, Meakins and Whiting (1955). The IR spectrometer used by these workers had an estimated maximum error of ±8 cm⁻¹, at 1700 cm⁻¹. In this table, the frequency of a band is followed by an intensity description.

<table>
<thead>
<tr>
<th>Isomer</th>
<th>C=O stretching band (cm⁻¹)</th>
<th>C=C stretching bands (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ME-CH\textsubscript{1}CH-CH\textsubscript{1}CH-CH\textsubscript{2}CO\textsubscript{2}-ME</td>
<td>1716 (850)</td>
<td>1642 (200) 1614 (100)</td>
</tr>
<tr>
<td>ME-CH\textsubscript{1}CH-CH\textsubscript{2}CH-CH\textsubscript{2}CO\textsubscript{2}-ME</td>
<td>1715 (700)</td>
<td>1639 (200) 1597 m</td>
</tr>
<tr>
<td>ME-CH\textsubscript{2}CH-CH\textsubscript{1}CH-CH\textsubscript{2}CO\textsubscript{2}-ME</td>
<td>1715 (800)</td>
<td>1634 (200) 1598 m</td>
</tr>
<tr>
<td>ME-CH\textsubscript{2}CH-CH\textsubscript{2}CH-CH\textsubscript{2}CO\textsubscript{2}-ME</td>
<td>1715 (650)</td>
<td>1623 (200) 1587 m</td>
</tr>
<tr>
<td>ME-CH\textsubscript{1}CH-CO\textsubscript{2}-ME</td>
<td>1726 (850)</td>
<td>1659 (180) -</td>
</tr>
<tr>
<td>ME-CH\textsubscript{2}CH-CO\textsubscript{2}-ME</td>
<td>1721 (800)</td>
<td>1644 (120) -</td>
</tr>
<tr>
<td>ME-CH=CH-CH\textsubscript{1}CH-CO\textsubscript{2}-ME</td>
<td>1719 (850)</td>
<td>1615 (200) -</td>
</tr>
<tr>
<td>ME-CH=CH-Ch\textsubscript{2}CH-CO\textsubscript{2}-ME</td>
<td>1730 (450)</td>
<td>1718 (250) 1612 (200)</td>
</tr>
<tr>
<td>ME-CH\textsubscript{1}CH=CH-Ch\textsubscript{2}CH-CO\textsubscript{2}-ME</td>
<td>1708 (550)</td>
<td>1626 (40) -</td>
</tr>
<tr>
<td>ME-CH\textsubscript{2}CH=CH-Ch\textsubscript{2}CH-CO\textsubscript{2}-ME</td>
<td>1713 (500)</td>
<td>1615 (30) -</td>
</tr>
</tbody>
</table>
Table 3.4.7.8.3; Positions of assumed C=C stretching bands observed in the spectrum of the 7th fraction from the methyl linoleate isomerisation experiment (see figure 2.4.3.11). Assignments were made by analogy with the methyl sorbate isomer results (Table 3.4.7.8.2).

<table>
<thead>
<tr>
<th>C=C band</th>
<th>position (cm⁻¹)</th>
<th>intensity</th>
<th>predicted isomer responsible for band</th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>1654</td>
<td>strong</td>
<td>remaining methyl linoleate isolated C=C bonds</td>
</tr>
<tr>
<td>b.</td>
<td>&lt; 1654</td>
<td>shoulder</td>
<td>C₂=C-C₂≡C (1642 cm⁻¹) or C₂≡C-C₂=C (1639 cm⁻¹)</td>
</tr>
<tr>
<td>c.</td>
<td>1625</td>
<td>v. weak</td>
<td>C₂=C-C₂≡C (1623 cm⁻¹)</td>
</tr>
<tr>
<td>d.</td>
<td>1612</td>
<td>medium</td>
<td>C₂=C-C₂=C (1614 cm⁻¹)</td>
</tr>
<tr>
<td>e.</td>
<td>1601</td>
<td>medium</td>
<td>C₂=C-C₂=C (1597 cm⁻¹)</td>
</tr>
<tr>
<td>f.</td>
<td>1582</td>
<td>medium</td>
<td>C₂=C-C₂≡C (1587 cm⁻¹)</td>
</tr>
</tbody>
</table>

3.4.7.9 Determination of the cis content of fats and oils using the cis C=C band and the CBS technique

A total of four fats and oils, selected on account of their different FAME compositions, were assayed for cis content to assess the feasibility of the CBS technique on real samples. The samples are listed below:

a. Trial fat (provided by the MAFF)
b. Fat B72E (provided by the MAFF)
c. Sunflower oil purchased from a local store
d. Gamma oil (oil of evening primrose)

These oils and fats were converted to the methyl esters by the potassium methoxide procedure (section 3.3.10.1). The compositions, determined by capillary GC (see section 2.3.4), are shown in Table 3.4.7.9.1 and summarised in Table 3.4.7.9.2. The latter table also shows the IR cis and trans content obtained by the CBS and straight baseline techniques respectively.
scanning and data processing procedures are shown in the table.

Examination of the FAME (liquid film) spectra produced from fat B72E and the trial fat (figures 3.4.7.9.1 and 3.4.7.9.2, respectively), showed the presence of an unassigned band (band x) at \( = 1580 \text{ cm}^{-1} \) which was not found in any of the spectra of FAME's studied thus far. The indications of the superimposed methyl stearate baseline and sample solute component spectra were that a poor fit was obtained for both fats (see Figures 3.4.7.9.3 and 3.4.7.9.4), owing to the unassigned band. However, the IR cis content results compared favourably with the GC results. This implies that the unassigned band does not overlap with the cis C=C band at the peak maximum, but probably interferes with the selection of the best baseline spectrum. An increase in noise level with decrease in sample concentration was noticed in the spectra obtained with a 2 mm pathlength. This accounts for the increase in difference between the cis contents determined by the IR and GC methods in solution (b) of both these fats.

**Table 3.4.7.9.1; Composition of the fats and oils determined by capillary GC.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>mono</th>
<th>di-</th>
<th>tri-</th>
<th>total</th>
<th>mono</th>
<th>di-</th>
<th>tri-</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>trial fat</td>
<td>12.82</td>
<td>0.26</td>
<td>-</td>
<td>13.08</td>
<td>35.48</td>
<td>6.87</td>
<td>-</td>
<td>42.37</td>
</tr>
<tr>
<td>B72E (a)</td>
<td>40.35</td>
<td>0.42</td>
<td>-</td>
<td>40.76</td>
<td>36.23</td>
<td>0.21</td>
<td>-</td>
<td>36.44</td>
</tr>
<tr>
<td>B72E (b)</td>
<td>39.22</td>
<td>0.23</td>
<td>-</td>
<td>39.45</td>
<td>37.49</td>
<td>0.04</td>
<td>-</td>
<td>37.53</td>
</tr>
<tr>
<td>sunflower oil</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>17.26</td>
<td>76.17</td>
<td>0.11</td>
<td>93.44</td>
</tr>
<tr>
<td>gamma oil</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>4.83</td>
<td>84.48</td>
<td>5.29</td>
<td>94.58</td>
</tr>
</tbody>
</table>

Where:  
mono = % of FAME's with one double bond  
di = % of FAME's with two double bonds  
tri = % of FAME's with three double bonds

**Note:** Trial fat and B72E had approximately 2% of 18:2\( \Delta \text{ct} \) and 18:2\( \Delta \text{tc} \), which were counted twice as mono cis and mono trans. This is why the total FAME percentage of these two fats are greater than 100%. Any unassigned FAME's were included in the percentage of saturated fat.
Table 3.4.7.9.2; IR and GC determination of the cis and trans content of various fats and oils.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sol.</th>
<th>Concentration g/5ml (CCl₄)</th>
<th>Position of Band cm⁻¹</th>
<th>IR Method</th>
<th>GC Method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>C=C  C=C-H 1 mm 2 mm</td>
<td>cis content %</td>
<td>trans content %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>cis  trans 1 mm 2 mm</td>
<td>cis content %</td>
<td>trans content %</td>
</tr>
<tr>
<td>B72E</td>
<td>(a)</td>
<td>0.402</td>
<td>1654 969 x 36.4</td>
<td>l. 41.5</td>
<td>Z</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>0.108</td>
<td>&quot; &quot; x 40</td>
<td>l. 40.4</td>
<td>Z</td>
</tr>
<tr>
<td>Trial fat</td>
<td>(a)</td>
<td>0.293</td>
<td>&quot; &quot; x 43.66</td>
<td>l. 12.6</td>
<td>Z</td>
</tr>
<tr>
<td></td>
<td>(b)</td>
<td>0.139</td>
<td>&quot; &quot; x 45.1</td>
<td>l. 13.3</td>
<td>Z</td>
</tr>
<tr>
<td>Sunflower</td>
<td>(a)</td>
<td>0.331</td>
<td>1656 - w w</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1649</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Gamma</td>
<td>(a)</td>
<td>0.211</td>
<td>1656 - w w</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1649</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Where:  
- x = the cis band was too noisy for accurate determination of the cis content.  
- z = the trans band absorbance was off scale and could not be measured.  
- w = the cis content was not determined because two overlapping cis C=C were present.  
- - = no trans band was present.  
- v = content was not determined by GC  
- sol. = solution

Note: Two different pathlength liquid cells were used to take the solvent, samples and the two sets of methyl oleate and methyl elaidate standards spectra, utilising the conditions described in section 3.3.2. A normalisation factor of 1 was used to subtract the solvent component from the spectra.

IR trans content determination:  
- Using straight baseline correction technique described in section 3.3.4.  
- Methyl elaidate standards used to produce calibration plot.

The following wavenumber parameters were used:  
- i. N₁ = 995 cm⁻¹  
- ii. N₁ = 981 cm⁻¹

IR cis content determination:  
- Using CBS technique described in section 3.3.5.  
- Methyl oleate standards were used.
The unassigned band was not observed in the spectrum of the FAME's produced from sunflower oil (fig. 3.4.7.9.5). Thus, the curved baseline technique fitted the carbonyl band profile very well (fig. 3.4.7.9.6). However, this unassigned band is present in the spectrum of gamma oil (fig. 3.4.7.9.7), and caused a poor fit of the baseline spectrum at the lower frequency end of the cis C=C band region (fig. 3.4.7.9.8). Two overlapping cis C=C stretching bands were found to be present in the spectra of gamma oil (FAME's) and sunflower oil (FAME's). The intensity of the lower frequency cis C=C band of gamma oil was greater than that of the corresponding band of sunflower oil (see figure 3.4.7.9.9). This could be due to the absorbance of band x in this region, or to the higher % of tri-unsaturated FAME's in gamma oil. The two cis C=C stretching bands observed in the spectra of gamma oil and sunflower oil, were due to the large percentage of di-unsaturated cis FAME's present in these oils. It was considered impracticable to assess the cis contents of these oils because of the overlapping cis C=C bands.

In contrast, fat B72E and the trial fat both contain very low levels of poly-unsaturated FAME's and a high percentage of mono-unsaturated FAME's. The cis content results obtained using the CBS technique (2 mm cell) and the more concentrated solutions were very similar to those obtained by the GC technique for these fats. Fat B72E contained 40% trans mono-unsaturated FAME's. This is illustrated by figure 3.4.7.9.10, which shows the spectrum in the region of the cis band. As expected the trans C=C stretching band (≈ 1668 cm⁻¹) is observed to overlap with the corresponding cis band. However, the contribution to the cis band at its maximum is negligible, and the cis content values determined by the IR and GC methods are very similar. These results verify the previous conclusions, regarding interference from the trans band. The other fats and oils either had little or no trans content, see Table 3.4.7.9.1; the straight baseline correction technique used to determine the trans content, produced results which were very close to those obtained by GC, regardless of the parameters used.

3.4.7.10 Repeatability measurements of the cis content by capillary GC and FTIR

Cis content repeatability experiments were conducted on fat N88 (provided by the MAFF) using the capillary GC method (see section 2.3.4) and the CBS FTIR method (see section 3.4.7.9). A comparative study of the KOH and TMAH methylation techniques was carried out at the same time (see explanatory notes, Table 3.4.7.10.2). Table 3.4.7.10.1 shows the cis mono-, cis di- and cis/trans di-unsaturated FAME content of the fat determined by the GC method. Included in
this table is the GC calculated IR cis percentage. This value takes into account the cis C=C band IR absorbances of the various classes of cis FAME's present (i.e. mono- = 1, di- = 1.5 and assume cis/trans di = 1), in giving an estimate of the expected IR cis % content (see Table 3.4.5.10.1 for details). The actual IR cis content results are shown in Table 3.4.7.10.2.

The overall mean IR cis content value calculated from the GC results (39.6 ± 0.8%) was close to the values obtained by the CBS IR method using 1 mm (41.1 ± 2.1%) and 2 mm (40.8 ±1.7%) cells. The cis band spectra obtained with the 1 mm cell were noticeably more noisy than those produced with the 2 mm cell. The coefficient of variance for the 2 mm cell results was twice that of the GC method, due mainly to poorly fitting baselines of some of the solute spectra (see figure 3.4.7.10.1). Eliminating the results obtained with poorly fitting baselines improved the overall coefficient of variance of the 2 mm cell results to 2.7%, making it broadly comparable to the GC results. Minor errors in weighings and dilutions could also be responsible for the larger coefficient of variance in the IR method, as these errors are not present in the GC method.

A considerable number of FAME's present at low levels were not assigned in the GC chromatograms, and these added up to levels as high as 5.7%, with an overall coefficient of variance of 20.8%. Most of the error with the GC method arose from the overlap of peaks.

From the above work it can be concluded that the repeatability of the IR method is as good as that of the GC method, provided that good baseline fits are obtained. No significant differences were apparent between the various methylation techniques from the GC results. Some differences were observed between the methylation sets of cis content results obtained using the IR method, but this was attributed to poor baseline fits rather than the methylation technique. It should be noted that the samples N88B001 to N88B004, prepared using an old batch of TMAH, required a period of 30 minutes to achieve complete methylation. In contrast methylation using a new batch of TMAH took several minutes. This emphasizes the need for TLC monitoring of the methylation.
Table 3.4.7.10.1; Cis content repeatability of the capillary GC method applied to the methyl ester of fat N88, prepared using two different methylation techniques.

<table>
<thead>
<tr>
<th>Solution</th>
<th>% mono cis</th>
<th>% di cis</th>
<th>% di cis/trans</th>
<th>unknown %</th>
<th>total GC % cis</th>
<th>cal. IR cis</th>
</tr>
</thead>
<tbody>
<tr>
<td>N88A001</td>
<td>38.4</td>
<td>0.34</td>
<td>1.1</td>
<td>2.74</td>
<td>39.8</td>
<td>40.2</td>
</tr>
<tr>
<td>N88A002</td>
<td>37.2</td>
<td>0.34</td>
<td>1.17</td>
<td>4.52</td>
<td>38.8</td>
<td>39.1</td>
</tr>
<tr>
<td>N88A003</td>
<td>38.6</td>
<td>0.30</td>
<td>1.08</td>
<td>4.17</td>
<td>40.0</td>
<td>40.3</td>
</tr>
<tr>
<td>N88A004</td>
<td>38.1</td>
<td>0.33</td>
<td>1.12</td>
<td>3.81</td>
<td>39.5</td>
<td>39.9</td>
</tr>
<tr>
<td>mean</td>
<td>38.1</td>
<td>0.33</td>
<td>1.12</td>
<td>3.81</td>
<td>39.5</td>
<td>39.9</td>
</tr>
<tr>
<td>std. dev.</td>
<td>±0.7</td>
<td>±0.02</td>
<td>±0.05</td>
<td>±0.94</td>
<td>±0.7</td>
<td>±0.7</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.9%</td>
<td>6.0%</td>
<td>4.5%</td>
<td>1.7%</td>
<td>1.7%</td>
<td></td>
</tr>
<tr>
<td>N88B001</td>
<td>36.9</td>
<td>0.33</td>
<td>1.17</td>
<td>4.49</td>
<td>38.4</td>
<td>38.7</td>
</tr>
<tr>
<td>N88B002</td>
<td>37.6</td>
<td>0.31</td>
<td>1.09</td>
<td>4.03</td>
<td>39.0</td>
<td>39.3</td>
</tr>
<tr>
<td>N88B003</td>
<td>37.5</td>
<td>0.32</td>
<td>1.08</td>
<td>5.72</td>
<td>38.9</td>
<td>39.3</td>
</tr>
<tr>
<td>N88B004</td>
<td>37.9</td>
<td>0.33</td>
<td>1.11</td>
<td>5.09</td>
<td>39.4</td>
<td>39.7</td>
</tr>
<tr>
<td>mean</td>
<td>37.5</td>
<td>0.32</td>
<td>1.11</td>
<td>4.83</td>
<td>38.9</td>
<td>39.2</td>
</tr>
<tr>
<td>std. dev.</td>
<td>±0.4</td>
<td>±0.01</td>
<td>±0.04</td>
<td>±0.73</td>
<td>±0.4</td>
<td>±0.4</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.2%</td>
<td>3.3%</td>
<td>3.6%</td>
<td>15.1%</td>
<td>1.0%</td>
<td>1.0%</td>
</tr>
<tr>
<td>N88BX01</td>
<td>39.5</td>
<td>0.31</td>
<td>1.09</td>
<td>2.82</td>
<td>40.9</td>
<td>41.2</td>
</tr>
<tr>
<td>N88BX02</td>
<td>38.1</td>
<td>0.31</td>
<td>1.11</td>
<td>4.42</td>
<td>39.5</td>
<td>39.8</td>
</tr>
<tr>
<td>N88BX03</td>
<td>36.3</td>
<td>0.34</td>
<td>1.13</td>
<td>3.53</td>
<td>39.7</td>
<td>40.1</td>
</tr>
<tr>
<td>N88BX04</td>
<td>37.5</td>
<td>0.33</td>
<td>1.11</td>
<td>3.66</td>
<td>38.9</td>
<td>39.2</td>
</tr>
<tr>
<td>N88BX05</td>
<td>36.3</td>
<td>0.33</td>
<td>1.12</td>
<td>3.74</td>
<td>37.7</td>
<td>38.1</td>
</tr>
<tr>
<td>N88BX06</td>
<td>39.0</td>
<td>0.31</td>
<td>1.07</td>
<td>3.32</td>
<td>40.3</td>
<td>40.7</td>
</tr>
<tr>
<td>N88BX07</td>
<td>37.1</td>
<td>0.32</td>
<td>1.14</td>
<td>3.6</td>
<td>38.5</td>
<td>38.9</td>
</tr>
<tr>
<td>N88BX08</td>
<td>38.5</td>
<td>0.35</td>
<td>1.16</td>
<td>3.33</td>
<td>40.0</td>
<td>40.3</td>
</tr>
<tr>
<td>N88BX09</td>
<td>37.3</td>
<td>0.32</td>
<td>1.10</td>
<td>4.14</td>
<td>38.7</td>
<td>39.1</td>
</tr>
<tr>
<td>N88BX10</td>
<td>38.2</td>
<td>0.33</td>
<td>1.11</td>
<td>2.87</td>
<td>39.7</td>
<td>40.0</td>
</tr>
<tr>
<td>mean</td>
<td>38.0</td>
<td>0.33</td>
<td>1.11</td>
<td>3.53</td>
<td>39.4</td>
<td>39.7</td>
</tr>
<tr>
<td>std. dev.</td>
<td>±1.0</td>
<td>±0.01</td>
<td>±0.03</td>
<td>±0.5</td>
<td>±0.9</td>
<td>±0.9</td>
</tr>
<tr>
<td>C.V.</td>
<td>2.5%</td>
<td>3.0%</td>
<td>2.7%</td>
<td>14.2%</td>
<td>2.4%</td>
<td>2.4%</td>
</tr>
</tbody>
</table>

Overall

| mean         | 3.89       | 39.31    | 39.6          |
| std. dev.    | ±0.81      | ±0.80    | ±0.8          |
| C.V.         | 20.8%      | 2.0      | 2.0           |

Notes:
- % unknown = % of FAME's which are unassigned
- total GC % cis = % mono cis + % di cis + % di cis/trans
- cal. IR cis % = this value gives an estimate of the expected total cis content determined by the IR technique, established by substituting the corresponding GC values into the following equation:
  - cal. IR cis % = % mono cis + % di cis/trans di + 1.5(% di cis)
- % mono cis = cis mono-unsaturated FAME content
- % di cis = cis di-unsaturated FAME content
- % tri cis = cis tri-unsaturated FAME content
Table 3.4.7.10.2; Cis content repeatability of the CBS IR method applied to the methyl ester of fat N88, prepared using two different methylation technique as described in Table 3.4.7.10.1. Overall (corrected) - statistics ignoring results with poor baseline fits.

<table>
<thead>
<tr>
<th>Solution</th>
<th>IR cis content %</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 mm cell</td>
<td>2 mm cell</td>
</tr>
<tr>
<td>N88A001</td>
<td>40.6</td>
<td>36.8*</td>
<td></td>
</tr>
<tr>
<td>N88A002</td>
<td>43.0</td>
<td>40.7</td>
<td></td>
</tr>
<tr>
<td>N88A003</td>
<td>41.2</td>
<td>42.0</td>
<td></td>
</tr>
<tr>
<td>N88A004</td>
<td>39.0</td>
<td>39.4</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>40.9</td>
<td>39.7</td>
<td></td>
</tr>
<tr>
<td>std. dev.</td>
<td>±1.6</td>
<td>±2.2</td>
<td></td>
</tr>
<tr>
<td>C.V.</td>
<td>4.0%</td>
<td>5.6%</td>
<td></td>
</tr>
<tr>
<td>N88B001</td>
<td>40.7</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>N88B002</td>
<td>39.3</td>
<td>41.9</td>
<td></td>
</tr>
<tr>
<td>N88B003</td>
<td>45.7</td>
<td>42.0</td>
<td></td>
</tr>
<tr>
<td>N88B004</td>
<td>41.4</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>41.8</td>
<td>41.6</td>
<td></td>
</tr>
<tr>
<td>std. dev.</td>
<td>±2.8</td>
<td>±0.4</td>
<td></td>
</tr>
<tr>
<td>C.V.</td>
<td>6.6%</td>
<td>0.9%</td>
<td></td>
</tr>
<tr>
<td>N88BX01</td>
<td></td>
<td>44.0</td>
<td></td>
</tr>
<tr>
<td>N88BX02</td>
<td>43.9*</td>
<td>42.3</td>
<td></td>
</tr>
<tr>
<td>N88BX03</td>
<td>38.3</td>
<td>37.8*</td>
<td></td>
</tr>
<tr>
<td>N88BX04</td>
<td>41.2*</td>
<td>38.9*</td>
<td></td>
</tr>
<tr>
<td>N88BX05</td>
<td>39.2*</td>
<td>42.4*</td>
<td></td>
</tr>
<tr>
<td>N88BX06</td>
<td>42.7*</td>
<td>40.5</td>
<td></td>
</tr>
<tr>
<td>N88BX07</td>
<td>42.2</td>
<td>40.8</td>
<td></td>
</tr>
<tr>
<td>N88BX08</td>
<td>42.2</td>
<td>41.1</td>
<td></td>
</tr>
<tr>
<td>N88BX09</td>
<td>40.6</td>
<td>40.4</td>
<td></td>
</tr>
<tr>
<td>N88BX10</td>
<td>37.4*</td>
<td>40.4</td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>40.9</td>
<td>40.9</td>
<td></td>
</tr>
<tr>
<td>std. dev.</td>
<td>±2.2</td>
<td>±1.8</td>
<td></td>
</tr>
<tr>
<td>C.V.</td>
<td>5.3%</td>
<td>4.3%</td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>41.1</td>
<td>40.9</td>
<td></td>
</tr>
<tr>
<td>std. dev.</td>
<td>±2.1</td>
<td>±1.7</td>
<td></td>
</tr>
<tr>
<td>C.V.</td>
<td>5.1%</td>
<td>4.2%</td>
<td></td>
</tr>
<tr>
<td>Overall (corrected)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean</td>
<td>41.2</td>
<td>41.3</td>
<td></td>
</tr>
<tr>
<td>std. dev.</td>
<td>±2.0</td>
<td>±1.1</td>
<td></td>
</tr>
<tr>
<td>C.V.</td>
<td>4.8%</td>
<td>2.7%</td>
<td></td>
</tr>
</tbody>
</table>

* = poor baseline fit
Notes applicable to both Tables 3.4.5.10.1 and 3.4.5.10.2.

The mean values, standard deviations and coefficient of variance are in bold type.

The following methylation procedures were used on the fat N88:
Methanolic potassium hydroxide (see section 3.3.10.1) - samples N88A001 to N88A004.
Tetramethyl ammonium hydroxide (see section 3.3.10.1) - samples N88B001 to N88B004 &
N88BX01 to N88BX10, using new and old batches of TMAH respectively. These methylation
techniques were monitored by TLC (section 3.3.11).

3.4.7.11 Collaborative study of biscuit fats

A total of seven powdered biscuits were provided by the MAFF to be analysed by the FTIR and
GC methods for the cis and trans FAME content of the fat composition. Samples of these
powdered biscuit were also sent to several other laboratories in the UK, with a brief to extract and
analyse the fat content in any manner they wished to adopt. This collaborative study was
coordinated by the MAFF to establish the variation in the results obtained by different
techniques in different laboratories. Biscuit fats were selected for the study because they
usually consist of hydrogenated vegetable oils or hydrogenated fish oils, which are known to
present lipid analysts with a significant challenge.

The biscuits are designated 7/15, 11/19, 4/6, 9/10, 13/17, 5/14 and 3/8. A Soxhlet extraction
technique (150 ml of diethyl ether, one hour) was used to extract the fats from each biscuit (7g).
Methylation was conducted according to the procedure described in section 3.3.10.2. Two
FAME solutions derived from each biscuit fat (0.2g/5ml) were prepared according to the
procedure described in section 3.3.12. The standard scanning conditions (50 co-added scans)
utilising 2 mm and 2.5 mm pathlength cell were used to produce the spectra. The IR cis C=C
stretching bands peak height absorbance values were established using the CBS method
described in section 3.4.5.9. Details of the calibration solutions and plots used to determine the
cis content from the C=C stretch band absorbances can be found in Table 3.4.7.11.1. The
capillary GC method used is described in section 2.3.4.

When the cis content results obtained with the IR method were compared with those obtained
by other laboratories there was poor agreement. GC results generated using the same solutions
used in the IR method, also showed poor agreement with those of the other laboratories.
Unfortunately, the present author was introduced into the study at a late date, and was supplied
with samples of the same brands of biscuits, but not the same batch. Therefore, the composition
of the fats in the different batches could have been dissimilar. This was confirmed by the MAFF laboratory, who reanalysed the samples provided and obtained similar results to those obtained by the author. The results obtained in the collaborative study are not quoted in this thesis.

Table 3.4.7.11.1: Cis content of various biscuit fats determined using the CBS IR method.

<table>
<thead>
<tr>
<th>biscuit fat</th>
<th>IR determined % cis content</th>
<th>cal. IR cis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>a</td>
<td>b</td>
</tr>
<tr>
<td>7/15 (a)</td>
<td>47.8\textsuperscript{L}</td>
<td>44.9\textsuperscript{L}</td>
</tr>
<tr>
<td>7/15 (b)</td>
<td>50.7</td>
<td>49.1\textsuperscript{L}</td>
</tr>
<tr>
<td>11/19 (a)</td>
<td>46.3</td>
<td>46.5</td>
</tr>
<tr>
<td>11/19 (b)</td>
<td>43.7\textsuperscript{h}</td>
<td>47.5</td>
</tr>
<tr>
<td>4/6 (a)</td>
<td>42.3\textsuperscript{h}</td>
<td>40.9</td>
</tr>
<tr>
<td>4/6 (b)</td>
<td>42.6\textsuperscript{h}</td>
<td>41.4</td>
</tr>
<tr>
<td>9/10 (a)</td>
<td>45.9</td>
<td>44.4</td>
</tr>
<tr>
<td>9/10 (b)</td>
<td>46.3</td>
<td>46.3</td>
</tr>
<tr>
<td>13/17 (a)</td>
<td>47.8\textsuperscript{h}</td>
<td>47.4\textsuperscript{h}</td>
</tr>
<tr>
<td>13/17 (b)</td>
<td>47.1\textsuperscript{h}</td>
<td>46.9</td>
</tr>
<tr>
<td>5/14</td>
<td>41.1</td>
<td>40.2</td>
</tr>
<tr>
<td>3/8</td>
<td>10.6</td>
<td>7.0\textsuperscript{L}</td>
</tr>
</tbody>
</table>

Where;

\begin{itemize}
  \item \textbf{a} = Results determined using calibration plot of methyl oleate. This plot was produced using the linear regression program traversing the origin. Both the calibration standards spectra and samples spectra were obtained using a 2 mm pathlength cell and the standard procedure.
  \item \textbf{b} = Identical to \textbf{a}, except spectra generated using a 2.5 mm pathlength cell.
  \item \textbf{c} = Results determined using calibration plot of methyl oleate and methyl stearate standards. This plot was generated using the linear regression program described in section 3.3.1.2. A 2.5 mm cell was used to produce the sample and standard spectra.
  \item \textbf{cal. IR cis} = Predicted cis content of IR technique calculated from GC data (see Table 3.4.7.11.2), using the following equation
    \[(\text{GC mono cis }\%) + 1.5(\text{GC di cis }\%) + 2.5(\text{GC tri cis }\%) + (\text{GC di-ch }\%)
  \]  
  \item \textsuperscript{+} = Noisy baseline spectrum, caused by over multiplication of baseline spectrum.
  \item \textsuperscript{h} = Poor baseline spectrum fit, causing the value to be high.
  \item \textsuperscript{L} = Poor baseline spectrum fit, causing the value to be low.
\end{itemize}
Table 3.4.7.11.2: Capillary GC analysis of biscuit fats (average of two results).

<table>
<thead>
<tr>
<th>FAME</th>
<th>% of each FAME determined by capillary GC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7/15</td>
</tr>
<tr>
<td>mono cis</td>
<td>34.9</td>
</tr>
<tr>
<td>mono trans</td>
<td>23.8</td>
</tr>
<tr>
<td>di-cis</td>
<td>4.7</td>
</tr>
<tr>
<td>di-trans</td>
<td>0.5</td>
</tr>
<tr>
<td>di-c/t</td>
<td>0.8</td>
</tr>
<tr>
<td>tri-cis</td>
<td>1.3</td>
</tr>
<tr>
<td>saturated</td>
<td>28.1</td>
</tr>
<tr>
<td>unassigned</td>
<td>6.0</td>
</tr>
<tr>
<td>unsaturated</td>
<td>66.0</td>
</tr>
<tr>
<td>cis unsat.</td>
<td>41.7</td>
</tr>
<tr>
<td>trans unsat.</td>
<td>24.3</td>
</tr>
</tbody>
</table>

Where:
- mono cis = total cis mono-unsaturated FAME
- mono trans = total trans mono-unsaturated FAME
- di cis = total cis di-unsaturated FAME
- di trans = total trans di-unsaturated FAME
- di-c/t = di-unsaturated FAME's with cis and trans bonds
- cis unsat. = total cis unsaturated content including di-c/t
- trans unsat. = Total trans unsaturated content including di-c/t

Table 3.4.7.11.1 shows the cis content of the biscuit fats determined using two calibration solutions and different pathlength cells. The results obtained using the 2 mm and 2.5 mm pathlengths were very similar in value. However, the baseline fit was generally poorer with the 2 mm pathlength spectra. The use of single or two-component calibration solutions was found to have no significant effect upon the results generated.

The percentage cis contents of the biscuit fats determined by the IR and GC methods were very different. This was attributed to the high percentage of FAME's which were unidentified by the GC technique (see Table 3.4.7.11.2), and the high content of cis di-unsaturated FAME's in the
biscuit fats. Included in Table 3.4.7.11.1 is the expected IR % cis content determined from the GC results. Good agreement was obtained between the IR and expected IR % cis content results for fats 7/15 and 11/19. However, very poor agreement was observed for the rest of the fats. This suggests that the method used to predict the IR values from the GC results is at fault.

3.4.7.12 CBS technique: further developments

Although the CBS baseline correction technique had considerable success at simulating the carbonyl shoulder on which the cis band lies, there were a number of cases where a poor fit was obtained. Attempts were made to improve the fit.

The most obvious way of increasing the number of good baseline fits was to increase the number of methyl stearate spectra in the library from which the baseline could be selected. To assess this, the cis C=C stretching absorbance was established for a set of methyl oleate calibration spectra using two CBS programs containing 12 and 18 methyl stearate baseline spectra respectively. Two techniques were used to measure the degree of success each CBS program had in fitting the baseline profile - visual and least-squares regression (see Table 3.4.7.12.1). Both these indicated that the larger the number of baseline spectra to choose from the greater the chance of a good baseline fit.

Table 3.4.7.12.1; linear regression results of the calibration plots of methyl oleate (cis C=C absorbance versus concentration of methyl oleate g/l). The second column lists the number of methyl stearate spectra from which the CBS program selected the best fitting baseline.

<table>
<thead>
<tr>
<th>Plot</th>
<th>no.</th>
<th>slope x10^{-3}</th>
<th>intercept x10^{-3}</th>
<th>std. dev. of points</th>
<th>correlation coefficient</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>12</td>
<td>5.554</td>
<td>-3.65</td>
<td>2.2 x10^{-3}</td>
<td>0.9996</td>
</tr>
<tr>
<td>B</td>
<td>18</td>
<td>5.454</td>
<td>-1.02</td>
<td>1.6 x10^{-3}</td>
<td>0.9996</td>
</tr>
</tbody>
</table>
The precision of the cis CBS method is also dependent upon the quality of the fit of the regression equation to the methyl oleate calibration data. In the work described above, a linear least-squares program which forced the line through the origin was used. However, after closer examination of the the plots it was apparent that they did not go through the origin. This clearly affects the precision of the method.

Sleeter et al. (1989) found that they could improve the precision of their IR method for determining the trans content by fitting a second order polynomial to the calibration data instead of the a straight line. Further increases in the precision were obtained when they used integrated band areas as a measure of the intensity of the band. Studies were conducted to investigate whether least squares linear (not forced through the origin) or quadratic fits of the calibration data would also improve the precision of the CBS technique. A program called AREA3.oy (see section 3.3.6) was written to calculate the cis C=C stretching band area, after the carbonyl shoulder had been subtracted from the spectrum using the CBS program. It should be noted that Sleeter et al. (1989) referred to the R² term as the correlation coefficient which is incorrect. R² is a measure, between 0 and 1, of the portion of the (corrected) total variation that is attributed to the fit, rather than left to residual error. It is the square of the multiple correlation coefficient, in other words, the square of the correlation between the dependent variable and the predicted value. It is called the coefficient of determination.

Table 3.7.12.2; Cis C=C peak absorbance data for a set of methyl oleate calibration spectra. x(obs) = concentration of methyl oleate (g/l), A = cis peak height determined using CBS program "SIM3.oy", B = cis peak integrated area determined using CBS area program "AREA3.oy" (N1 = 1663 cm⁻¹ & N2 = 1643 cm⁻¹).

<table>
<thead>
<tr>
<th>Sol.</th>
<th>x (obs)</th>
<th>Cis peak absorbance values</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>a.</td>
<td>3.48</td>
<td>1.85E-2</td>
<td>0.14068</td>
<td></td>
</tr>
<tr>
<td>b.</td>
<td>6.96</td>
<td>3.74E-2</td>
<td>0.27087</td>
<td></td>
</tr>
<tr>
<td>c.</td>
<td>13.91</td>
<td>7.24E-2</td>
<td>0.54134</td>
<td></td>
</tr>
<tr>
<td>d.</td>
<td>20.87</td>
<td>11.36E-2</td>
<td>0.81417</td>
<td></td>
</tr>
<tr>
<td>e.</td>
<td>27.82</td>
<td>15.22E-2</td>
<td>1.0939</td>
<td></td>
</tr>
<tr>
<td>f.</td>
<td>34.78</td>
<td>18.78E-2</td>
<td>1.352</td>
<td></td>
</tr>
</tbody>
</table>

168
Cis C=C band height and area data generated from a set of methyl oleate calibration spectra is shown in Table 3.4.7.12.2. Least squares linear and quadratic fits generated from this data are illustrated in Table 3.4.7.12.3, which also shows the error in the coefficients, correlation of determination ($R^2$) and the standard error of estimate. This latter term was used by Sleeter et al. (1989) as a predictor of the accuracy of the analysis, and is the root mean square average of the deviations between the predicted line and the actual data.

### Table 3.4.7.12.3: Results of linear and quadratic regressions of data in Table 3.4.7.12.2

| Model: Linear regression using cis C=C band height data |
| Equation: $\frac{\% \text{ cis}}{\text{cis}} = -1.0164E^{-3} + 5.454E^{-3} \times \text{Height}$ |
| Std. dev.: $\pm1.2E^{-3}, \pm5.9E^{-5}$ |
| $R^2 = 0.9995$; standard error of estimation = $1.60E^{-3}$ |

| Model: Linear regression using cis C=C band area data |
| Equation: $\frac{\% \text{ cis}}{\text{cis}} = 2.576E^{-3} + 3.893E^{-2} \times \text{AREA}$ |
| Std. dev.: $\pm4.1E^{-3}, \pm1.9E^{-3}$ |
| $R^2 = 0.9999$; standard dev. of points = $5.22E^{-3}$ |

| Model: Quadratic regression using cis C=C band height data |
| Equation: $\frac{\% \text{ cis}}{\text{cis}} = -8.59E^{-4} + 5.429E^{-3} \times \text{Height} + 6.788E^{-7} \times \text{Height}^2$ |
| Std. dev.: $\pm2.31E^{-3}, \pm3.02E^{-4}, \pm7.81E^{-6}$ |
| $R^2 = 0.9995$, standard dev. of points = $1.85E^{-3}$ |

| Model: Quadratic regression using cis C=C band area data |
| Equation: $\frac{\% \text{ cis}}{\text{cis}} = 1.30E^{-3} + 3.914E^{-2} \times \text{Area} - 5.50E^{-6} \times \text{Area}^2$ |
| Std. dev.: $\pm7.49E^{-3}, \pm9.79E^{-4}, \pm2.53E^{-5}$ |
| $R^2 = 0.9999$, standard dev. of points = $5.99E^{-3}$ |

The linear fit of the band height data gave a correlation of determination of 0.9995 and a standard error of estimate of $1.6x10^{-3}$% cis. Using the band area data improved the correlation of determination (0.999) for the linear regression but produced a poorer value for the standard error of estimate ($5.2x10^{-3}$%). In the case of the quadratic models for the height and area data, the correlation of determination values were identical to those of their respective linear models. However, the standard errors of estimation were both slightly higher than the respective values obtained by the linear models. This was totally unexpected, and suggests that linear models ($y=mx+c$) with the peak height data produce the most precise results.
3.4.7.13 Comparison of cis C=C peak height and area CBS techniques on samples high in either trans or cis poly-unsaturated FAME's

In the previous section simple peak height and peak area experiments on calibration data of the cis C=C band were conducted. These showed that the CBS method produces the most precise results when linear models with peak height data were used. In this section, the effects of peak height and peak area measurements have on real sample results were studied. These were compared against results generated by the capillary GC and IR (cis =C-H band) methods.

The standard conditions were used to obtain the solution and solvent spectra (section 3.3.2). A normalisation of one was used to subtract the solvent component. Details of the integration parameters, cell pathlength and calibration solutions used in each of the IR methods are reported in Table 3.4.7.13.2. Also shown in this table are the IR predicted values calculated using GC composition results from Table 3.4.7.13.1, and the cis content values determined using the cis =C-H stretching band. The fats (FT001 to FT004) were selected for this experiment because they contained high trans or high cis poly-unsaturated contents. Each of the IR methods will be discusses separately:

Method A (C=C peak height CBS technique); FT001 and FT002 both contained low levels of cis poly-unsaturated FAME's and as expected the predicted IR cis contents, determined from the GC results, were very similar to the GC cis unsaturated content values. Both these values were lower than those determined by the IR technique for each fat. This was attributed to the high levels of unidentified FAME's in the GC technique. The appreciably higher coefficient of variance (C.V.) observed for FT001 was attributed to an outlier caused by a poor baseline fit.

Significant differences were observed between the predicted IR and GC results of fats FT003 and FT004, due to the high levels of cis poly-unsaturated FAME's. Once again, the IR determined results were higher than the predicted IR results determined from the GC values.

Method's B and C (C=C peak area measurements using different baseline parameters); On first impressions this technique gives values which are comparable to the GC established cis content values, thus compensating for the differences in absorbance of cis polyunsaturated FAME's. However, examination of the solute spectra revealed that it was due to errors in the area measurements from two sources; (a) interference of the trans C=C band, as
observed for FT001 and FT002 (fig. 3.4.7.13.4). (B) cis C=C band broadening caused by high levels of cis poly-unsaturated FAME's, as observed for FT003 and FT004 (fig. 3.4.7.13.5).

Table 3.4.7.13.1: Capillary GC analysis of fats (as methyl ester) assayed by MAFF laboratory.

<table>
<thead>
<tr>
<th>FAME</th>
<th>% of each FAME determined by capillary GC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FT001A</td>
</tr>
<tr>
<td>mono cis</td>
<td>28.4</td>
</tr>
<tr>
<td>mono trans</td>
<td>38.2</td>
</tr>
<tr>
<td>di-cis</td>
<td>0.5</td>
</tr>
<tr>
<td>di-trans</td>
<td>0.8</td>
</tr>
<tr>
<td>di-c/t</td>
<td>0.9</td>
</tr>
<tr>
<td>tri-cis</td>
<td>0.2</td>
</tr>
<tr>
<td>saturated</td>
<td>19.3</td>
</tr>
<tr>
<td>unassigned</td>
<td>11.8</td>
</tr>
<tr>
<td>unsaturated</td>
<td>68.9</td>
</tr>
<tr>
<td>cis unsat.</td>
<td>29.9</td>
</tr>
<tr>
<td>trans unsat.</td>
<td>39.9</td>
</tr>
</tbody>
</table>

Abbreviations from Table 3.4.5.13.1
- mono cis = total cis mono-unsaturated FAME
- mono trans = total trans mono-unsaturated FAME
- di cis = total cis di-unsaturated FAME
- di trans = total trans di-unsaturated FAME

Abbreviations continued from Table 3.4.5.13.1
- di-c/t = di-unsaturated FAME's with cis and trans bonds
- cis unsat. = total cis unsaturated content including di-c/t
- trans unsat. = Total trans unsaturated content including di-c/t

FT001B is the same trial fat FT001A except analysed on later date.
Table 3.4.7.13.2; Cis % content of various trial fats (used in collaborative study) determined by various IR methods.

<table>
<thead>
<tr>
<th>Trial fat</th>
<th>Cis content % by IR methods</th>
<th>predicted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>method A</td>
<td>method B</td>
</tr>
<tr>
<td>FT001a.</td>
<td>32.8</td>
<td>28.1</td>
</tr>
<tr>
<td>b.</td>
<td>34.1</td>
<td>27.2</td>
</tr>
<tr>
<td>c.</td>
<td>32.5</td>
<td>27.3</td>
</tr>
<tr>
<td>d.</td>
<td>32.1</td>
<td>27.3</td>
</tr>
<tr>
<td>e.</td>
<td>33.2</td>
<td>27.2</td>
</tr>
<tr>
<td>mean</td>
<td>33.1</td>
<td>27.0</td>
</tr>
<tr>
<td>std. dev.</td>
<td>1.4</td>
<td>0.5</td>
</tr>
<tr>
<td>C.V.</td>
<td>4.3%</td>
<td>1.9%</td>
</tr>
<tr>
<td>FT002 a.</td>
<td>38.3</td>
<td>34.4</td>
</tr>
<tr>
<td>b.</td>
<td>37.8</td>
<td>35.0</td>
</tr>
<tr>
<td>c.</td>
<td>38.1</td>
<td>34.9</td>
</tr>
<tr>
<td>d.</td>
<td>38.9</td>
<td>35.3</td>
</tr>
<tr>
<td>e.</td>
<td>39.0</td>
<td>34.9</td>
</tr>
<tr>
<td>mean</td>
<td>38.4</td>
<td>34.9</td>
</tr>
<tr>
<td>std. dev.</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.3%</td>
<td>0.9%</td>
</tr>
<tr>
<td>FT003 a.</td>
<td>117.1</td>
<td>-</td>
</tr>
<tr>
<td>b.</td>
<td>118.3</td>
<td>-</td>
</tr>
<tr>
<td>c.</td>
<td>116.3</td>
<td>-</td>
</tr>
<tr>
<td>d.</td>
<td>119.7</td>
<td>-</td>
</tr>
<tr>
<td>e.</td>
<td>118.3</td>
<td>-</td>
</tr>
<tr>
<td>mean</td>
<td>117.9</td>
<td>-</td>
</tr>
<tr>
<td>std. dev.</td>
<td>1.3</td>
<td>-</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.1%</td>
<td>-</td>
</tr>
<tr>
<td>FT004 a.</td>
<td>113.0</td>
<td>88.0</td>
</tr>
<tr>
<td>b.</td>
<td>111.3</td>
<td>88.0</td>
</tr>
<tr>
<td>c.</td>
<td>111.1</td>
<td>87.2</td>
</tr>
<tr>
<td>d.</td>
<td>112.0</td>
<td>87.1</td>
</tr>
<tr>
<td>e.</td>
<td>109.9</td>
<td>87.0</td>
</tr>
<tr>
<td>mean</td>
<td>111.5</td>
<td>87.4</td>
</tr>
<tr>
<td>std. dev.</td>
<td>1.2</td>
<td>0.5</td>
</tr>
<tr>
<td>C.V.</td>
<td>1.0%</td>
<td>0.6%</td>
</tr>
</tbody>
</table>

Notes for Table 3.4.7.13.2-

Method A = peak height of cis C=C band (used 2.5 mm cell/ CBS baseline correction/ methyl oleate calibration spectra to produce linear plot)

Notes continue on following page.
Method B = cis C=C band area (used: - 2.5 mm cell/ CBS baseline correction followed by AREA3.oy (parameters N1 = 1663 cm⁻¹, N2 = 1643 cm⁻¹, see figure 3.4.7.13.1) methyl oleate calibration spectra used to produce linear plot)

Method C = cis C=C band area (used: - 2.5 mm cell/ CBS baseline correction followed by AREA3.oy (parameters N1 = 1669 cm⁻¹, N2 = 1640 cm⁻¹, see figure 3.4.7.13.2) methyl oleate calibration spectra used to produce linear plot)

Method D = cis =C-H band peak height (used: - 2.5 mm cell/ straight baseline correction method (parameters N1 = 3036 cm⁻¹, N2 = peak maximum, N3 = 2986 cm⁻¹, see figure 3.4.7.13.3) methyl oleate calibration spectra used to produce linear plot)

predicted = predicted IR cis content established from the GC results on Table 3.4.7.13.1 using the equation on Table 3.4.7.10.1 (called cal. IR cis %). Values in brackets represent levels of unidentified FAME's by GC method.

Method D (=C-H stretching band peak height measurements); Using this technique, significantly lower values were obtained than those established by the GC technique or the other IR techniques. This was owing to the errors associated with the use of a straight baseline correction technique on a band occurring on the shoulder of a larger band. This was further complicated by minor bands attributed to trans and saturated FAME's.

To summarise, the experimental results in this section show that errors caused by the overlap of bands, assigned to saturated and trans FAME's, make the use of the =C-H stretching band unreliable for quantitation of the cis content. In the case of the C=C band peak height CBS technique, trans or saturated FAME's, were not found to interfere with quantitation. Unfortunately, cis poly-unsaturated FAME's cause errors in both methods using the cis C=C and =C-H bands peak height measurements, due to the overlapping of bands. In contrast, poly-unsaturated cis FAME's as well as trans FAME's were found to cause errors in the C=C band area CBS technique. From these results it can be concluded that the cis C=C peak height CBS technique will produce the most accurate cis content results provided the level of cis poly-unsaturated FAME's is very low in the sample studied. Cis poly-unsaturated FAME's cause errors in all three methods studied.
3.4.8 Quantitation of isolated cis di- and tri-unsaturated FAME’s

One of the major problems with quantitative IR spectroscopy is the inconvenience of overlapping bands, which either obscure altogether the band of interest or overlap sufficiently to create an error in the absorbance value of the band of interest. The measurement of the absorbance of the band at 913 cm\(^{-1}\) is representative of the latter case. This band is present in the spectra of the cis isolated di- and tri-unsaturated FAME’s, but has not been assigned. Obviously the quantitative measurement of this band would enable the determination of these types of FAME, and have the additional bonus of allowing the cis isolated FAME content to be established in samples with a high poly-unsaturated content, by applying corrections to the absorbance in either the cis C=C or =C-H stretching regions.

From the experience gained from the cis C=C band measurement, it was apparent that the straight baseline correction technique could not be applied to the 913 cm\(^{-1}\) band. Unsuccessful attempts were made to simulate the overlapping bands in this region by using the spectrum of methyl stearate in a similar fashion to the cis C=C band problem (see SIM4.cyx program). The problems were due to the large variations in absorbance and number of overlapping bands observed for FAME’s differing in cis/trans configuration, position of double bond or chainlength (see section 2.4.5).

Some success was achieved using a cubic spline program which simply fits a cubic equation between two points but takes into account any other cubic equation between adjacent points. The net effect is a smooth curved line which fits a profile provided by a series of points. Figure 3.4.8.1 shows the overlapped spectra of methyl linoleate and the simulated cubic spline baseline in the region 1000 to 800 cm\(^{-1}\). The cubic spline spectrum was produced by entering the absorbance values of the corresponding wavenumber regions, 1000 (N1) to 940 cm\(^{-1}\) (N2) and 895 (N3) to 880 cm\(^{-1}\) (N4), thus missing out the data containing the cis peak. From this the cubic spline program then fits cubics between the data points to produce a smooth curve, thus simulating a baseline. Experiments using the spectra of methyl stearate and methyl linoleate suggested that poor simulations are obtained if the wavenumber parameters used to calculate the cubic spline (N1 to N2 and N3 to N4) are kept constant. A recommendation is made of a potential solution to this problem in section 3.5.
3.5 CONCLUSIONS

Studies conducted in this chapter have focused on the determination of the cis and trans content of fats and oils by means of the quantitative measurement of various IR bands under controlled conditions. IR bands utilised for this purpose include the following:

**Band A**: trans =C-H out of plane deformation vibration (~ 969 cm⁻¹)

**Band B**: cis =C-H stretching vibration (~ 3005 cm⁻¹)

**Band C**: cis C=C stretching vibration (~ 1654 cm⁻¹)

**Band D**: unassigned band at 913 cm⁻¹ observed in di- and tri-unsaturated FAME's with cis isolated double bonds.

A number of quantitative methods have been published utilising the first two bands above, which have been described in detail in the literature review. Current investigations on these two bands attempted to resolve several issues established from the literature review. No methodology has been reported using the bands C and D. This is primarily due to the difficulties associated with quantitation of low intensity bands, complicated by the interference of overlapping bands. FTIR was found to offer a greater signal-to-noise ratio (by addition of spectra) and spectral manipulation, hence facilitating the quantitation of such bands. Results obtained using these IR methods were compared against those produced by a capillary GC method. Each of the bands A to D will be discussed separately:

**Band A**

From the literature review of the methodology using the trans =C-H out-of-plane deformation vibration (~ 969 cm⁻¹) two issues were established. These were, (a) the uncertainty of the capability of the baseline correction techniques (used to compensate for the absorbance of overlapping bands) and (b) the influence of trans FAME's isomers possessing different positional and chainlengths upon the trans content value, which is calculated as a percentage weight of methyl elaidate present.

Studies conducted on model mixtures and samples of hydrogenated fats and oils, revealed that the straight baseline correction technique with fixed wavenumber parameters, failed to compensate for the absorbance of overlapping bands present in cis or saturated FAME's. The
use of different sets of fixed wavenumber parameters on the sample and calibration spectra did not resolve the problem. Modifications to this method were found to improve the accuracy, these include the use of a two-component set of calibration spectra and variable straight baseline parameters. These changes were found to compensate for the band broadening of the trans band with increase in trans content. In order for this method to work for all samples, the assumption was made that the profiles of the interfering bands were the same for both cis and saturated FAME's in the region of the trans band, regardless of chainlength, number of double bonds or position of double bonds. Studies conducted using methyl elaidate, and either methyl oleate or methyl stearate, as the two-component solutions produced similar results, confirming the assumption. However, studies in Chapter 2 on FAME's revealed differing band profiles in this region for cis poly-unsaturated and saturated FAME's, which varied in either the number of double bonds or the chainlength. Further investigations are recommended to assess the effects of these band profiles changes upon the baseline applied and thus the % trans content values. Repeatability experiments using peak height and area measurements, from five repeat samples, produced good coefficients of variation which were comparable to the GC results.

Overall, the differences in results observed between the IR methods conducted showed the necessity for a new standardised IR method which uses a defined two-component set of calibration solutions, and outlines the mode of establishing the variable baseline parameters. For example, the baseline could be drawn onto the sample spectra by superimposing the most appropriate calibration spectrum with the baseline drawn on it. Alternatively, an algorithm could be used to establish the wavenumber minima on either side of the trans band.

Research into the extinction coefficient of the trans band at of various trans mono-unsaturated FAME's, established from the plot of peak height absorbance versus concentration (moles/litre), showed a small increase in the value with increase in chain length of the FAME's. The extinction coefficient of the isomer 18:1Δ6t was almost identical to that of 18:1Δ9t. However, the value for 18:1Δ11t was lower than that of 18:1Δ9t, but the purity of this sample was questionable. A notable exception to the trend outlined above was observed for 18:1Δ9t12(OH), where the hydroxy bands interfered with the trans band determination. A totally different picture is obtained when the extinction coefficient is expressed in terms of grams/litre. Here, there is a significant increase in the value of the extinction coefficient with decrease in the molecular weight of the trans FAME. In the case of positional isomers, no significant differences were obtained between the Δ6 and Δ9 positional isomers. This was not the case for the Δ11 isomer, due to the reasons outlined previously. When grams/litre are used, the implications of the above
on fats and oils with a high percentage of only one trans FAME, other than 18:1Δ9t, is that the %
trans content will be in error because results are reported in terms of the percentage weight of
18:1Δ9t. The degree of error is dependent on the chainlength of the trans FAME and the
quantity present. For example, in the extreme case that 14:1Δ9t is the only trans FAME present
in a sample, then the trans content will be significantly higher (16%) than the true value. On the
other hand, in a sample containing only 22:1Δ13t, the trans content value will be significantly
lower than the true value (14%).

In practice fats and oils containing a high percentage of trans content, have in general a
distribution range for the carbon number of trans monoenes C14 to C24, with the greatest
proportion of trans FAME’s in the C18 range. Thus the error attributed to the differences in the
extinction coefficients of various chainlength trans FAME’s when concentrations are expressed
in g.l⁻¹, are likely to be cancelled out. Positional isomers in the range Δ6 to Δ9 do not affect the
trans value. However, there is still uncertainty regarding the influence of the positional isomers in
the Δ9 to Δ11 range upon the trans value, but it is expected to produce little or no error. Poly-
unsaturated trans FAME’s containing isolated or conjugated double bonds also absorb at 969
cm⁻¹ and are known to create an error.

In terms of the criteria used to assess the different methods, the following may be concluded
from these experiments;

**Specificity:** The following were observed to cause errors in the trans peak height (or area)
absorbance measurement due to interfering band or bands;

(A) Saturated and cis unsaturated FAME’s.
(B) Trans monoenes isomers of differing chainlength, and possibly positional isomers.
(C) Poly-unsaturated conjugated and non-conjugated trans FAME’s.

Out of this list, only the poly-unsaturated non-conjugated trans FAME’s and those described in
(B), can not be corrected for.

**Accuracy:** The accuracy of the trans content results is dependent upon the levels of FAME’s
present in the sample which are known to interfere with the measurement of the trans band. For
example, samples with trans components consisting predominantly of monoenes with a
distribution range of carbon numbers C16 to C20 and positional isomers Δ6 to Δ11, the level of
error in the IR trans content calculation will be low. Samples containing high levels of trans polyenes and trans monoenes FAME's, outside this distribution range, will produce a repeatable error in the trans content results. This may be higher or lower than the true trans value depending upon the composition. Clearly, the application of two-component calibration solutions, and variable wavenumber parameters for the straight baseline, reduce the errors attributable to the cis and saturated FAME's.

**Sensitivity;** This is dependent upon the calibration solutions and wavenumber parameters used. Levels as low as 1% may be easily determined using a two-component calibration solution and variable wavenumber parameters for the selection of the straight baseline.

**Precision;** Repeatability experiments comparing the capillary GC and FTIR techniques, showed that the coefficients of variability of the trans content results are comparable, when the levels of unidentified FAME's in the GC chromatograms are taken into account. Reproducibility experiments were not conducted, but the FTIR methods would be expected to give better results than the capillary GC methods, provided a standard FTIR method is followed. Factors such as degradation of liquid phase, unidentified FAME's, overlap of FAME peaks and the use of different liquid phase cause the capillary GC results to be less reproducible between laboratories.

**Robustness;** The establishment of standardised FTIR method with a protocol to determine the variable wavenumber parameters would enhance robustness.

Further studies are recommended into the following:-

A. The extinction coefficient of the trans band of positional isomers of 18:1Δ9t and their influence upon the % trans content value.

B. To establish whether the overlapping bands, in the trans region (1100 to 900 cm⁻¹), possess the same profile for cis poly-unsaturated FAME's and different chain length cis and saturated FAME's. Then to assess the effect these overlapping bands have upon the IR trans method applying the straight baseline correction technique.

C. A re-investigate of the quantitative studies conducted by Kaufmann et al. (1959), which involved the IR measurement of Ba salts of fatty acids in KBr discs. This is considered
appropriate because the interfering bands attributed to cis and saturated FAME's in the trans region are not observed and the problems limiting the use of this method could easily be overcome using FTIR in place of a conventional dispersive instrument (see section 3.2.1).

D. Investigate the potential quantitative use of Raman active bands (e.g. the cis and trans C=C stretching bands).

**Band B**

Preliminary studies on the cis -C-H stretching vibration extinction coefficient of various cis FAME's confirmed the findings reported in the literature, that the intensity of this band is related to the number of isolated cis double bonds present in the molecule. Analysis of model mixtures utilising cis -C-H band measurements, by means of a tangent method, produced poor agreement between the known and determined cis content values. This was attributed to the absorbance of bands on either side of the cis band, which are present in both saturated and trans FAME's. Moreover, errors were introduced by the use of the tangent method which consists of a straight baseline drawn on the shoulder of a strongly absorbing band. When real fats and oils were assayed using this method, samples high in trans FAME's gave significantly lower cis content values than the corresponding capillary GC method, verifying the conclusions reached on the model mixtures. In contrast, levels greater than 100% cis content were obtained using this IR method on samples containing high levels of cis poly-unsaturated FAME's, arising due to the linear relationship between the intensity of the cis -C-H band and the number of cis double bonds. From these results it can be concluded that the cis -C-H band has little or no application for the determination of the cis content. However, it can be used to estimate the total number of cis double bonds in samples high in poly-unsaturated FAME's, provided the levels of saturated or trans unsaturated FAME's are low. It was noted that the frequency of the cis -C-H stretching band increases with increase in the degree of cis unsaturation.

In terms of the criteria used to assess the different methods, the following may be concluded from these experiments:-

**Specificity:** The list on the following page were observed to cause errors in the cis peak height absorbance measurements due to interfering band or bands:-
(A) Saturated and trans unsaturated FAME's.
(B) Poly-unsaturated conjugated and non-conjugated FAME's.

None of these interfering FAME's can be corrected for in the present method.

**Accuracy:** This is dependent upon two factors, (a) the quantities of FAME's present in the sample which are known to interfere with the measurement of cis band (category (A) under specificity) and (b) the quantities of cis poly-unsaturated FAME's present, which have cis band extinction coefficient greater than one. This clearly limits the application of this method to samples which have an appreciable amount of cis mono-unsaturated FAME's present.

**Sensitivity:** This is once again dependent upon the levels of the interfering FAME's present in the sample. In model mixtures of methyl oleate and methyl stearate the cis $\equiv$C-H band could still be detected at the 2% level, however, the difference between the known and determined value for this solution was approximately 12%.

**Precision:** Repeatability experiments show that the coefficient of variability of the cis content results generated by this method are comparable to those produced by other IR methods and the GC method. Reproducibility experiments were not conducted.

**Robustness:** This method should have a high degree of robustness.

Further studies are recommended into the following:-

(A) To establish whether the frequency differences observed between various cis $\equiv$C-H bands corresponding to the cis mono-, di- and tri-unsaturated FAME's, are sufficient to allow resolution and therefore quantitation of the various cis categories.

(B) To assess whether spectral subtraction techniques can be used to eliminate the interfering bands attributed to trans and saturated FAME's.
A novel method of determining the cis content was developed by the author utilising the cis \( \text{C} = \text{C} \) stretching band at 1654 cm\(^{-1} \), which lies on the shoulder of the strongly absorbing carbonyl stretching band at 1742 cm\(^{-1} \). Preliminary experiments using a straight baseline correction technique to determine the cis peak height were unsuccessful due to the curvature of the carbonyl shoulder and the interference of the corresponding trans \( \text{C} = \text{C} \) band which overlaps slightly. The application of a methyl stearate spectrum, which contains no \( \text{C} = \text{C} \) stretching bands, to simulate the curvature of the carbonyl band was found to provide a satisfactory baseline. However, the profile of the carbonyl band was found to change with concentration. To overcome this problem a program was written which selected the best fitting methyl stearate spectrum from a library of spectra. After this selection, the program subtracts the methyl stearate spectrum from the solution spectrum, before calculating the cis \( \text{C} = \text{C} \) peak height absorbance.

Summarized below are the criteria used to validate this method, based on studies conducted on the relationship between the intensity of the cis \( \text{C} = \text{C} \) stretching band and the position/ molecular weight of cis mono- and poly-unsaturated FAME's. Also those results generated from model mixtures and real fats and oils.

**Specificity:**

1. Cis \( \text{C} = \text{C} \) band studies showed that little or no change is observed in the intensity when methylene groups are added or removed from the methylene chains \( X \) or \( Y \) on either side of the double bond, provided that the number of methylenes are not less than 6 and 3 in the \( Y \) and \( X \) chains respectively. Studies into the effects of decreasing the numbers of methylenes on either side still further were not conducted, but the likelihood of the terminal groups, in particular the carbonyl group, affecting the cis \( \text{C} = \text{C} \) band intensity increases.

\[
\text{CH}_3-(\text{CH}_2)_y-\text{CH}=\text{CH}-(\text{CH}_2)_x-\text{COOCH}_3
\]

The introduction of a hydroxy group into the molecule reduced the intensity of the band and was attributed to hydrogen bonding between the two groups. Errors in the cis content results determined in terms of the weight of methyl oleate are likely to occur if there is a molecular weight distribution of cis monoenes other than 18:1, for the same reasons outlined for trans FAME's, see section on band A.
2. The trans FAME's C=C stretching band is observed in the IR at ≈ 1670 cm⁻¹, but at a significantly lower intensity than the corresponding band for the cis FAME's. The overlap between the C=C stretching bands of cis and trans FAME's causes little or no error in the peak height absorbance measurement, whereas the same cannot be said of area absorbance measurement. Enhancement or deconvolution programs may be used to resolve these bands.

3. Cis poly-unsaturated FAME's with isolated double bonds also possess C=C bands which overlap with the corresponding cis monoene band. Furthermore, the intensities of these bands in cis poly-unsaturated FAME's are greater than that of the corresponding cis monoenes, however, they are not equivalent to the number of isolated cis double bonds present. Errors are therefore present in the % cis content when cis poly-unsaturated FAME's are present. Estimations of these errors have been calculated in section 3.4.7.7.

4. Conjugated isomers of methyl linoleate with the configurations trans-trans, cis-trans and trans-cis all possess C=C bands which overlap with the cis isolated C=C stretching band. The presence of these conjugated isomers is therefore expected to introduce an error in the cis % content determined by the CBS FTIR technique.

Accuracy; The accuracy of the cis content results is dependent upon the presence of FAME's listed under specificity. These cause errors in the cis content results. For example, analyses of samples high in poly-unsaturated conjugated or non-conjugated FAME's are likely to have low accuracy.

Sensitivity; Studies conducted on model mixtures achieved the quantitation of a cis FAME present at a 2% level. This level may be reduced still further if a more stringent baseline simulation selection protocol is utilised.

Precision; Comparison of GC and IR repeatability experiments showed that the coefficient of variance for the IR results (4.2%) was twice that of the GC results (2.0%). Three sources of error, poor baseline fits, weighting of sample and dilutions of solutions, are present in the IR method but not in the GC method, and therefore potential causes of the high coefficient of variance of the IR method. Eliminating the results obtained with poorly fitting baselines significantly improved the overall coefficient of variance for the IR results (2.7%). From these results it can be concluded that the use of a more rigorous baseline selection protocol would achieve IR
repeatability results which are equivalent to those of the GC method. It is worth noting that the GC results did not take into account the varying quantities of unidentified FAME's. No reproducibility experiments were conducted, but a similar argument to that used for the IR method used to determine the trans content could apply.

**Robustness/simplicity;** The FTIR method is considered to be more robust than the capillary GC methods available, because the following variables are not present:

- batch to batch variability between liquid phase used on GC columns.
- variability between different liquid phases used.
- decomposition of liquid phase.
- expertise required by operator to obtain optimum resolution and interpret the GC results.

In the case of this FTIR method, the spectra were obtained and then processed using a program which provides solute and baseline spectra superimposed if requested in addition to the a numerical value for the cis peak height absorbance. All the analyst need do is visually examine the solute and superimposed baseline spectra to ensure a good baseline fit is obtained. This goodness of fit may be interpreted by a program, hence the FTIR method could be used by an untrained operator.

**Further studies are recommended into the following:-**

A. To evaluate the extinction coefficients of the cis band of positional isomers outside the range studied in this thesis and determine the influence upon the % cis content value.

B. To establish whether the spectral differences observed for the cis C=C stretching bands of mono-, di- and tri-unsaturated FAME's can be resolved and used quantitatively.

C. To isolate and conduct quantitative studies on the various conjugated isomers of methyl linoleate and establish their influence upon the % cis content value determined from the cis C=C band.

D. To refine the program used to obtain the methyl stearate simulated baselines.
A method was partially developed which could be used to measure the band at 913 cm$^{-1}$, characteristic of cis di- and tri-unsaturated FAME's with isolated double bonds. A cubic spline program was used to simulate the baseline spectrum in this method. However, experiments showed that poor simulations were obtained if the wavenumber parameters for the cubic spline were kept constant. Further work is recommended on the use of derivative spectra in the selection of wavenumber parameters. This would work on the principle that the derivative spectrum enhances bands, and hence facilitates the selection of the start and end wavenumbers required for the cubic spline program.

Investigations are also recommended into deconvolution or least squares curved fitting techniques. These may allow resolution of the band at 913 cm$^{-1}$ and hence quantitation. The deconvolution technique has been described in some detail in section 2.3.8. This technique is generally used to establish the position of bands, but has been used by Willis et al. (1984) to determine the length of the methylene sequence in ethylene propylene copolymers, in order to establish their physical and chemical properties. The least squares curved fitting technique has the most potential because it is capable of resolving a series of overlapping bands.

**Summary**

The accuracy of the FTIR method utilising the band at 969 cm$^{-1}$ is dramatically impaired if significant levels of the following trans FAME's are present in a fat sample: -

- conjugated or non-conjugated poly-unsaturated trans FAME's.
- positional trans monoene isomers outside the range $\Delta 6$ to $\Delta 11$.
- or when the predominant trans FAME is not 18:1.

All or some of these FAME's are likely to occur in highly hydrogenated fats or oils, which means that the accuracy of the results obtained by the FTIR method is questionable. In addition, issues regarding the standardisation of a trans IR method need to be resolved. In the case of the FTIR method utilising the cis C=C band to establish the cis content, samples containing significant levels of cis poly-unsaturated or conjugated isomer are considered unsuitable, because they introduce a repeatable error in the accuracy of the results obtained. The absence or presence of
the band at 913 cm$^{-1}$ can be readily used to establish whether the cis poly-unsaturated FAME levels are significant.

Assuming a standardised method can be established for the FTIR trans content determination, then the FTIR methods used to determine either cis or trans content have strengths and weaknesses which complement one another. For example, in highly hydrogenated samples where all the trans isomers likely to create an error in the result are present, the levels of cis poly-unsaturated will probably be negligible allowing accurate cis content results to be obtained. In the case of samples high in poly-unsaturated FAME’s the trans IR method would be used. There are, of course circumstances when significant errors will be present in both the IR methods. For example, when high levels of conjugated isomers are present. These conjugated isomers can easily be established from their IR spectra by their characteristic bands in the region 1000 to 800 cm$^{-1}$. One of the major drawbacks of these FTIR methods is that they provide little information about the composition of the sample, which is easily achieved using capillary GC. Having said this, the capillary GC method does have significant problems, which have been highlighted in the introduction and verified in the experimental sections. Hence, neither the FTIR methods nor the capillary GC method alone can accurately quantitate complex mixtures of FAME’s such as highly hydrogenated oils fish. To accomplish this a combination of the two techniques is required.
4.1 INTRODUCTION

The quantitative determination of the trans and cis fatty acid content of fats and oils using various IR bands and GC was reviewed in Chapter 3. All the methods discussed had inherent disadvantages, for example with the capillary GC technique, difficulties were encountered in resolving and identifying samples containing complex mixtures of isomers. For the IR techniques, the difficulty involved the elimination of interfering bands, and the absorbance of identical classes of polyene fatty acids at the same frequency.

To overcome these difficulties a new technique is required. Such a method should separate the components of the complex mixtures frequently encountered, as well as identify and quantify individual components. The application of GC-FTIR to this problem was seen as a logical extension of this project, because it clearly offers great potential for the separation and characterisation of components of the complex mixtures found in foodstuffs in general. The usefulness of the GC-FTIR technique in the determination of trans-fatty acid content will be assessed in this chapter and compared with methods which are already established. A short literature review of the application of GC-FTIR to lipid analysis is given first.

4.2 LITERATURE REVIEW

This review will only cover aspects of the application of GC-FTIR to the analysis of fats and oils. The author recommends the reader to a book by Herres (1987) which gives a digestible account of the theory and applications of GC-FTIR.

The first publication to refer to the analysis of FAME's by GC-FTIR technique was by Mantz (1977).
It consisted of a chromatogram showing various FAME peaks and their respective vapour phase spectra. His apparent objective was to show potential applications for GC-FTIR on behalf of his company who manufactured these instruments. No attempt was made to interpret the spectra. Examination of the spectra by the present author suggested that the sample studied was probably an oil, because no trans bands were present in any of the spectra. Furthermore, the cis \(-\text{C-H} \) stretching band was noticed in the spectra.

Erickson (1979) published a review on the applications of GC-FTIR to the energy industry. He described the identification of various short chain saturated FAME's extracted from water produced during coal gasification. An excellent example of the application of GC-FTIR to the elucidation of geometric configurations was the analysis of termite trail pheromones by Yamaoka et. al. (1987). These are generally straight chain aliphatic compounds with several double bonds. Although these compounds are in a different class from fats and oils, the presence of cis and trans isomers makes the results relevant. The number and positions of the double bonds were determined by GC-MS on acetylated derivatives, and by partial hydrogenation of isomers. The double bond configurations in the partially hydrogenated isomers (separated by capillary GC) were determined by the presence or absence of the trans band at 970 cm\(^{-1}\). By combining the GC-MS and GC-FTIR data, the termite trail pheromone structure was determined as cis-3, cis-6, trans-8-dodecatrien-1-ol. It was interesting to note that the partially hydrogenated isomer cis-6, trans-8-dodecadionyl acetate possessed two trans bands at 976 cm\(^{-1}\) and 949 cm\(^{-1}\) which were visible in the vapour phase spectrum. This is in agreement with spectral data reported in Chapter 2 for conjugated cis, trans bonds.

Sebedio et al. (1987) combined GC-MS and GC-FTIR data to establish the structure of cyclic fatty acid mixtures isolated from heated linseed and sunflower oils. They were the first to report the spectra of methyl oleate and methyl elaidate in the vapour phase. The detected differences between cis and trans isomers in the vapour phase are listed below and were used to characterise the unidentified FAME's.
The corresponding bands of a cis ethylenic bond in a 6 membered ring were at 660 cm\(^{-1}\) and 3034 cm\(^{-1}\) for the vibrations described above. Using this information they were able to distinguish between a cis ethylenic bond in a 6 membered ring and a straight carbon chain. Finally, a recent paper by Mossoba et al. (1990) confirms many of the present author's conclusions regarding the use of GC-FTIR for the quantitative analysis of fats and oils. This paper describes the use of capillary gas chromatography/matrix isolation/FTIR spectroscopy to identify C\(_{18}\) FAME isomers from hydrogenated soybean oil and margarines. Furthermore, FAME's containing trans double bonds were quantified. The matrix isolation/FTIR system works by cryogenically trapping the GC effluent in a microscopic solid argon matrix at 11 K, for subsequent analysis by FTIR. This facility enables several hundred interferograms to be co-added, thus allowing spectra with good signal to noise ratios to be obtained for samples quantities corresponding to 0.9 ng deposited on the Cryolect disk. Mossoba et al. also established correlations between certain bands, which were independently determined by the present author and will be discussed in the sections that follow. Further details of Mossoba's paper will be discussed in the final conclusion.
4.3 EXPERIMENTAL

4.3.1 Heated gas cell

Description

A schematic drawing of the RIIC GH-9 heated gas cell is given in figure 4.3.1.1. The body is made of 18/8 stainless steel which is tapered internally to reduce unnecessary volume. At each end, two silicone rubber O-rings are fitted inside rectangular grooves. KBr windows are held over the silicone rubber O-rings by means of metal caps each secured by four Allen screws. The path length of the cell was 9 cm. Heating was provided by heating elements incorporated into the cell body, the temperature of which could be controlled by adjusting the power supply from a Variac. This instrument also possessed a high temperature vacuum valve and two ports. One of these ports was used for the injection of the sample, the other allowed the passage of the thermocouple (chromel-alumel in a stainless steel sheath) used to monitor the temperature of the cell.

Experimental procedure

The gas cell was purged with dry oxygen free nitrogen to remove any water, carbon dioxide and oxygen according to the following procedure:

4.3.1A The gas cell was evacuated and then filled with nitrogen three or four times, while at room temperature, to ensure all the air had been replaced. At the end of the process the vacuum tap was closed.

4.3.1B Following the initial purging procedure, the cell was slowly heated to 200°C before being re-purged using the procedure described above. This process was repeated several times to remove any water and oxygen remaining on the interior surface of the cell.

After conducting these purging procedures the cell was allowed to cool to room temperature before being placed in the sample holder of the instrument while still connected to the Variac. A long needle syringe was used to deposit 10 microlitres of methyl elaidate on the bottom of the cell.
without spraying the windows. The instrument lid was closed and sealed with clingfilm (to prevent air from entering) and the instrument and sample compartment then purged with nitrogen for approximately fifteen minutes before the heating and the program SCANTIME.oy were started simultaneously. The program SCANTIME.oy allowed scans to be taken at set time intervals, and stored these on a floppy disc for future reference (see details in Appendix II). The heating was controlled manually by adjusting the Variac. The cell temperature was increased from room temperature to 200 °C. Listed below are the conditions entered into the program SCANTIME.oy.

- Resolution - 2 cm⁻¹
- Number of scans co-added per spectrum - 5
- Scanning mode - ratio (used the initial spectrum as the background spectrum)

### 4.3.2 GC-FTIR

**Description**

A schematic diagram of the Perkin Elmer GC-FTIR system set-up is shown in figure 4.3.2.1, the salient features of which are described below.

1. **Capillary gas chromatograph**

   The instrument used was a Carlo Erba HRGC 5300 mega series flame ionization gas chromatograph equipped with a split injector. A hole was drilled in the oven wall to accommodate the heated transfer line of the IR detector. The general arrangement used for capillary column GC is shown in figure 4.3.2.2. The capillary column is mounted at one end to the injector splitting system and at the other to the output splitter assembly, which splits the effluent into two fractions, of which 5 to 10 percent goes to the FID detector while the remainder travels down the transfer line.

2. **Transfer line**

   The transfer line consists of three meters of fused silica tubing of 0.32 mm internal diameter. The
line has negligible volume and is accurately temperature controlled to prevent condensation. The transfer line conveys the effluent to the light pipe.

3. Light pipe

The IR cell follows the conventional light pipe design. It is a gold-coated quartz tube of 570 µl volume, with coated KBr windows. The flow arrangements shown in Figure 4.3.2.3, allow gases to either pass through or to be held in the cell. This is done without the use of valves (which are always prone to contamination) between the column and the cell. A facility is also provided for the supply of make-up gas, whenever the finite volume of the light pipe may affect the resolution of the peaks in the infrared chromatogram. The temperature of the light pipe is accurately controlled.

4. Detectors

Two IR detectors were installed in the spectrometer; a deuterated triglycine sulphate (DTGS) and a more sensitive narrow band mercury cadmium telluride (MCT) detector (4000 to 700 cm⁻¹). These detectors could not be used simultaneously and the latter was not received until the final stages of this project.

5. Collection of data

The GC-FTIR accessory was used to obtain IR spectra from the eluents of a gas chromatograph. These are used to generate an IR chromatogram.

Infrared chromatograms were generated by the PACGRAM (Pseudo Auto Correction Chromatogram) method originally suggested by Haseth and Lephart. The chromatogram is derived from the interferograms after subtracting the interferogram corresponding to the background generated at the start of a run. Fifty interferogram points on either side of zero path difference are used, starting after the first five points on either side. The dot product of these two sets of points is formed and the square root of this is plotted to give the chromatogram. The response is claimed to be linear with concentration, provided that the sample transmittance is above 70%. As the signal generated is directly related to transmittance a linear relationship with concentration should not be expected for more strongly absorbing samples.

A small Spectrum computer was integrated into the FTIR system, in order to obtain, store and analyse the GC chromatograms produced. The computer and programs for this system were kindly
provided by Dr. G. J. Buist. Data collection on both the Spectrum computer and GC-FTIR accessory was started by means of a switch installed on the gas chromatograph.

6. Control of GC-FTIR

The accessory is operated with additional soft key controls on the model 1710 intelligent controller and with controls on the GC-IR control module. When the accessory is fitted, the following operations can be selected:

a. Setting periods for the collection of spectral data during the acquisition of a chromatogram.

b. Setting the instrument to obtain spectra either from peaks that are above a selected infrared threshold in the IR chromatogram, or after a delay period following each GC instrument chromatogram peak which is above a selected threshold.

c. Storing the average of the series of spectra obtained across a peak.

d. Dividing a peak into time slices, where each slice comprises of one or more scans, and storing the average spectrum for the spectra in each slice.

e. Closing the light pipe to hold a fraction of the eluate corresponding to a chromatogram peak. Spectra can be obtained and averaged for the held peak to improve the signal to noise ratio. When the pipe is closed the succeeding eluents by-pass the pipe.

4.3.3 Calculation of mean noise

The noise program provided by Perkin Elmer performs a statistical analysis of the ordinate data in a defined flat spectral region where no peaks are present. It calculates the mean of the ordinate values, the peak-to-peak (P-T-P) and the root mean square (RMS) noise in the region defined. The P-T-P noise is the difference between the minimum and maximum ordinate values from a fitted line, while the RMS noise refers to the RMS of variance of the observed ordinate values from the line.

An OBEY program called NOISE.oy was written by the author, incorporating the Perkin Elmer program, to establish the RMS and P-T-P noise for the four main regions listed below:-
The obtained RMS and P-T-P noise for each region were used to calculate the standard deviations of the band ratios. This can be done because the established absorbance of a band has an error (standard deviation) equivalent to the noise of points or regions on either side of the band used to draw the baseline. For example, in the calculation of the C-H/C=O band ratio standard deviation, it is necessary to establish the noise of the C-H peak absorbance measurement, which is the average of the noise in regions 1 and 2 above (see equation 4.3.3.1). In a similar manner the standard deviation of the C=O absorbance measurement is the average of the noise in regions 3 and 4 (see equation 4.3.3.2). Hence, the standard deviation of the C-H/C=O band ratio is the ratio or average of the standard deviation (std. dev.) established for the individual bands (see equation 4.3.3.3).

Equation 4.3.3.1; Average noise (either RMS or P-T-P) or std. dev. of the C-H stretching band absorbance.
\[ N_5 = \sqrt{(N_1^2 + N_2^2)/2} \]

Equation 4.3.3.2; Average noise (either RMS or P-T-P) or std. dev. of the C=O stretching band absorbance.
\[ N_6 = \sqrt{(N_3^2 + N_4^2)/2} \]

Equation 4.3.3.3; Average noise (either RMS or P-T-P) or std. dev. of the C-H/C=O stretching band ratio absorbance.
\[ N_7 = \sqrt{(N_5^2 + N_6^2)/2} \]

The abbreviations used in the equations represent the following:
- \( N_1 \) = noise of region 1 (either RMS or P-T-P, value obtained from program)
- \( N_2 \) = noise of region 2 (either RMS or P-T-P, value obtained from program)
- \( N_3 \) = noise of region 3 (either RMS or P-T-P, value obtained from program)
- \( N_4 \) = noise of region 4 (either RMS or P-T-P, value obtained from program)
- \( N_5 \) = Std. dev. (noise) of C-H band absorbance
- \( N_6 \) = Std. dev. (noise) of C=O band absorbance
- \( N_7 \) = Std. dev. (noise) of the C-H/C=O band ratio absorbance
Standard deviation of band ratios for plots in this chapter were calculated using equation 4.3.3.4, which were found to give more realistic results than that provided by equation 4.3.3.5.

\[
\text{equation 4.3.3.4} = \frac{(C-H \pm N7)}{C=O} \\
\text{equation 4.3.3.5} = (C-H/C=O) \pm N7
\]

4.4 RESULTS AND DISCUSSIONS

4.4.1 Vapour phase spectrum of methyl elaidate

No publications on the vapour phase spectra of unsaturated FAME's were found in preliminary literature searches. Therefore before substantial financial outlay was committed to the purchase of a Perkin Elmer GC-FTIR accessory, it was necessary to assess whether significant differences were observed between the spectra of different FAME's in the vapour phase. In order to achieve this aim, a vapour phase spectral sampling technique was developed using the heated gas cell described in section 4.3.1. This was used to take the vapour phase spectrum of methyl elaidate using the experimental procedure in section 4.3.1. This technique was developed as a result of initial unsuccessful experiments using the various procedures described below.

In the first set of experiments undertaken, the interleaved mode of the FTIR was utilised. This in effect allows the instrument to run as a double beam spectrometer. A motor is used to switch the sample holder in and out of the infrared beam, thus taking the background and sample spectra alternately. This ensures that the sample spectra are ratioed against recently run background spectra. Unfortunately this procedure was found to have two problems; firstly, the movement of the shuttle in and out of the infrared beam caused rotation of the cell and thus changed the position of the rectangular apertures of the caps holding the KBr windows in place. The nett effect was that different quantities of radiation traversed the cell. Secondly, although the sample compartment was purged with nitrogen, using the procedure 4.3.1A, it was impossible to eliminate all the atmospheric absorption bands, and these were very apparent in the spectra produced. To overcome these problems the ratio mode was used. In this mode the cell does not move and stays in the infrared beam throughout the experiment. A spectrum of the purged cell at
room temperature (containing methyl elaidate carefully deposited) taken at the start of the experiment is used as the background, which is ratioed against the spectra produced during the heating process. Interfering atmospheric compounds were found to diffuse out of the steel surface with increase in temperature. The elaborate procedure described in section 4.3.1b was found to minimise the interference from these compounds in the spectra produced.

Figure 4.4.1.1 shows the vapour phase spectrum of methyl elaidate obtained using the final procedure. Interfering water and CO₂ bands are just visible in this spectrum, which were eliminated by further spectral subtraction. Figure 4.4.1.2 represents the resultant vapour phase spectrum of methyl elaidate. This spectrum was found to be almost identical to the spectrum of methyl elaidate in the liquid phase. From this result it was concluded that vapour phase spectra could be used to distinguish trans, saturated and cis FAME's, and that the purchase of the GC-FTIR accessory was justifiable.

4.4.2 Preliminary GC-FTIR experiments using the DTGS detector

The experiments described in this section were conducted to establish the feasibility and limitations of the DTGS IR detector when used in combination with a high-performance narrow bore capillary column in the GC-FTIR system.

Two FAME mixtures (A) and (B) (see table 4.4.2.1) were analysed using a 50 metre 0.32 mm diameter column coated with 0.4 microns of CP Sil88 liquid phase. A split capillary injection system was used to inject the mixtures into the column. At the exit of the column the FID sampling split flow was diverted, the remaining 87% of the column flow passing to the infrared light pipe. This was calculated from flow rate measurements at the FID and FTIR exits. The operating conditions used are shown in table 4.4.2.1.

In the preliminary experiments 0.4 microlitres of the each mixture were injected into the column using a split ratio of 100:1, which is generally used in the standard capillary GC method. The IR chromatogram showed no peaks above the level of the noise for either mixture, except for the solvent. In the case of the corresponding FID chromatograms, the correct number of resolved peaks were observed. Spectra obtained using either the IR or the FID trigger consisted of noise for both mixtures. Spectra of FAME's were obtained when spectral acquisition was triggered manually over the whole chromatogram. This however required several runs of the same sample.
because the spectral capacity (64 spectra) was insufficient to cover the whole chromatogram of either sample. The reasons for the failure of both the IR and FID triggers to acquire spectral data are discussed in section 4.4.7.2. Examination of the spectra produced for each mixture showed only the presence of the strongest FAME bands, which were only just visible above the noise, on one or two spectra from each set. From these results it was clear that greater sensitivity was required.

Table 4.4.2.1: GC-FTIR operating conditions

**GC:**
- oven temperature = 195 °C
- injector temperature = 250 °C
- detector temperature = 250 °C
- 87% of the eluent leaving the end of the column goes to the light pipe (this value was calculated from the flow rate measurements at the FID and IR exits)
- flow rate through light pipe = 1.75 ml/min
- flow rate through bottom split = 8.4 ml/min
- top split closed (A splitless injection mode was used)

**IR:**
- Resolution = 8 cm⁻¹
- gain = 4
- make-up gas through light pipe = N₂
- number of accumulated (co-added) background spectra = 16
- transfer line and light pipe temperature = 200 °C
- data acquisition sensitivity on IR chromatogram (IR trigger) = 25 units

Solutions used:
- Mixture A = methyl oleate (16.044g/l, 90.7%) + methyl elaidate (1.6357g/l, 9.3%) in CCl₄
- Mixture B = mixture of saturated methyl ester (C₈, C₁₀, C₁₂, C₁₄, C₁₆) in octane (conc. of methyl esters unknown)

In order to obtain a FAME peak response (or spectrum) on the IR chromatogram, the quantity of sample reaching the light pipe had to be increased, this was achieved by reducing the split ratio. However, this had the adverse effect of overloading the column which reduces the column efficiency and thus the resolution between peaks (in particular between cis/trans isomers). A compromise was obtained using a split ratio of approximately 4:1. Table 4.4.2.2 shows the results
used to produce the Van Deemter plot and the retention times of methyl oleate and methyl elaidate (mixture (A)), which were used to obtain the optimum conditions using this split ratio.

Table 4.4.2.2; Data used to produce Van Deemter plot. Mixture (A) was used to generate this data.

<table>
<thead>
<tr>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
<th>U</th>
<th>N</th>
<th>H</th>
<th>Tma</th>
<th>Tmb</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.9</td>
<td>2.2</td>
<td>10</td>
<td>4.5</td>
<td>38.8</td>
<td>4142</td>
<td>1.096</td>
<td>87.5</td>
<td>85</td>
</tr>
<tr>
<td>1</td>
<td>2.6</td>
<td>11</td>
<td>4.2</td>
<td>42</td>
<td>14374</td>
<td>0.32</td>
<td>81.5</td>
<td>78.5</td>
</tr>
<tr>
<td>1.1</td>
<td>3</td>
<td>12</td>
<td>4</td>
<td>45.2</td>
<td>10076</td>
<td>0.45</td>
<td>75.6</td>
<td>73.3</td>
</tr>
<tr>
<td>1.15</td>
<td>3.3</td>
<td>12.9</td>
<td>3.9</td>
<td>47.2</td>
<td>6981</td>
<td>0.65</td>
<td>71</td>
<td>68.8</td>
</tr>
<tr>
<td>1.2</td>
<td>3.5</td>
<td>14</td>
<td>4</td>
<td>48.8</td>
<td>6836</td>
<td>0.66</td>
<td>68.5</td>
<td>66.5</td>
</tr>
</tbody>
</table>

Note:—

- **A** = inlet pressure
- **B** = light pipe flow rate
- **C** = bottom split flow rate
- **D** = split ratio
- **U** = average linear gas velocity (methane 195 °C)
- **N** = number of theoretical plates (methyl oleate peak used in calculation)
- **H** = height equivalent to one theoretical plate
- **Tma** = retention time of methyl oleate
- **Tmb** = retention time of methyl elaidate

Figures 4.4.2.1 and 4.4.2.2 show the IR and FID chromatograms, respectively, of mixture (A). The FID chromatogram shows two partially resolved peaks which have considerable tailing due to column overload. The IR chromatogram on the other hand, shows only the presence of a weak peak, this may be due to either;

1. The quantity of methyl elaidate is below the level detectable by the DTGS detector.
2. Closely eluting cis/trans peaks merging in the light pipe.

The best vapour phase spectrum obtained for mixture (A) (figure 4.4.2.3) was found to resemble that of the liquid phase spectra of cis FAME's. Table 4.4.2.3 shows the frequencies and assignments of relevant bands in this spectrum. The IR and FID chromatograms of mixture (B) are shown in Figures 4.4.2.4 and 4.4.2.5 respectively. The FAME peaks were resolved in the FID
chromatogram but not in the IR chromatogram. These observations suggested that make-up gas was required.

Table 4.4.2.3; Frequencies and assignments of bands observed in the GC-FTIR spectrum of mixture A (see figure 4.4.2.3). Note that the bands in the finger print region were not assigned.

<table>
<thead>
<tr>
<th>frequency cm⁻¹</th>
<th>band assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>ca. 3010</td>
<td>cis =C-H stretch</td>
</tr>
<tr>
<td>ca. 2940</td>
<td>asymmetric C-H stretch</td>
</tr>
<tr>
<td>ca. 2870</td>
<td>symmetric C-H stretch</td>
</tr>
<tr>
<td>ca. 1760</td>
<td>C=O stretch</td>
</tr>
<tr>
<td>ca. 1180</td>
<td>C-O skeletal coupled with α -CH₂- deformation</td>
</tr>
<tr>
<td>ca. 720</td>
<td>methylenes C-H rock and possible cis =C-H deformation</td>
</tr>
</tbody>
</table>

Attempts to use make-up gas were unsuccessful due to an irregular flow rate caused by a faulty gauge. All the spectra obtained resembled those of saturated FAME's in the liquid or solution phase and could be easily distinguished from the vapour phase spectrum of methyl oleate by the absence of the cis =C-H band at ca. 3010 cm⁻¹. Figure 4.4.2.6 shows the vapour phase spectrum of C₁₄, which is representative of the spectra obtained for this mixture.

Decreasing the quantity of mixture (A) reaching the light pipe by injecting less than 0.4 microlitres, or by using a split injection mode, drastically affected the quality of the best FAME spectrum. In fact only the strongest bands could be observed over the noise in the spectra. From this it was concluded that the quantity of sample reaching the light pipe under the conditions described represents the minimum quantity of methyl oleate required to produce a recognisable spectrum when a DTGS IR detector is used. The calculations below show how this quantity of methyl oleate was determined. It should be stressed though that this value is an approximation because it is assumed that the methyl elaidate peak has not merged with that of methyl oleate in the light pipe and that 87% of the eluent reaching the end of the column is transferred to the light pipe (this figure is based on data generated at a different pressure and temperature).
EXAMPLE CALCULATION

Relevant data used;

Concentration of methyl oleate in solution = 16.04g/l
Minimum syringe volume required to produce a recognisable spectrum = 0.4 μl
Flow through light pipe = 1.75 ml/min
Bottom split flow rate = 8.4 ml/min
Top split closed
Percentage flow at end of column that flows past FID to light pipe = 87%

Calculation;

The flow rate through the column = flow rate through light pipe + flow rate through FID

\[ \text{Flow rate through light pipe} = \frac{1.75 \times 100}{87} = 2.0 \text{ ml/min} \]

Hence, % of sample entering column = \( \frac{2}{(8.4 + 2) \times 100} \times 100 \) = 19.2%

Quantity of sample that reaches the light pipe form the end of the column (87%)

\[ \frac{19.2 \times 87}{100} = 16.7\% \]

Thus, 16.7% of the sample injected reaches the light pipe

Quantity of sample injected = \( \frac{16.044 \times 1 \times 10^3}{1} \times 0.4 \times 6.42 \times 10^{-6} \text{g} = 6.42 \mu\text{g} \)

Quantity reaching light pipe = \( \frac{6.42 \times 10^{-6} \times 16.7}{100} \times 1.07 \times 10^{-6} \text{g} = 1.07 \times 10^{-6} \text{g} = 1070 \text{ng} \)

From this calculation it was shown that the minimum quantity of methyl oleate required to produce a recognisable spectrum using the DTGS IR detector is about 1000 ng. This data demonstrates that this IR detector lacks the sensitivity to detect the small quantities of samples which are eluted form a high-performance capillary column. Spectra of eluted compounds could be obtained but only at the expense of resolution. The successful use of these columns has been reported in various papers (Shafer et al., 1980 and 1981; Crawford et al., 1982; Smith, 1984 and 1985; Jakobsen et al., 1982; Wilkins et al., 1982; Griffiths et al., 1983; Herres et al., 1985), where high resolution of components was required. All of these papers report using a mercury cadmium telluride (MCT) detector, which is much more sensitive than a DTGS detector.
4.4.3 Sensitivity of GC-FTIR system using MCT IR detector

As a result of the findings in the previous section an MCT detector was purchased and installed in the GC-FTIR system to improve the sensitivity. To provide a qualitative assessment of sensitivity the following experiment was conducted.

The reported sensitivities for commercially available GC-FTIR systems are in the low nanogram region. The following solutes have been used as sensitivity standards by Herres (1987), who obtained recognisable spectra of 20 ng m-cresol, 9 ng isobutyl methacrylate (IBMA, most commonly used sensitivity standard for GC-FTIR), 100 ng of toluene and 10 ng of N,N-dimethyl formamide (DMF). The vapour phase spectra reported resembled closely the corresponding liquid phase spectra, but some differences occur in the relative intensities of the bands.

Solutions of the above mentioned standards were accurately made up using chloroform as the solvent and injected into the GC-FTIR system under the conditions outlined below. The spectra of IBMA, toluene and m-cresol were not obtained due to overlap or poor resolution with the solvent peak. This was due to differences in the retention properties of the column used in our laboratory and that use by Herres, who used a 30m x 0.32mm i.d. fused silica DB-1, 0.25um film.

The spectrum of 14.8 ng of DMF was successfully obtained using the GC-FTIR system (see figure 4.4.3.1). The quantity of DMF reaching the light pipe was established by taking into account all the split ratios of the flow as described previously. Comparison of the DMF spectrum with that produced by Herres (figure 4.4.3.2), show that the latter has a considerably higher signal to noise ratio. This is probably due to a combination of a smaller eluent volume and the light pipe volume (100μl). Notwithstanding this higher noise level evident in the authors result, it was concluded that the GC-FTIR system used had sufficient sensitivity to detect amounts of eluents down to approximately 10 ng.

Experimental conditions used

Column: 50 m / 0.32 i.d. / carrier gas H₂ / liquid phase CP Sil88 - thickness 0.4 microns.
Instrument: oven temp. = 140 °C / injector & detector temp. = 250 °C.
Split ratio ((top + bottom split flow):(IR flow + FID flow)) = 9:1
Split ratio ((top flow + bottom flow + FID flow):(IR flow)) = 18:1
IR: resolution = 8cm⁻¹ / no make-up gas / gain = 1 / no. of accumulated background spectra = 16 / transfer-line and light- pipe temp. = 200 °C / data acquisition sensitivity = 20 units
4.4.4 Discussion of IR chromatograms

The IR chromatogram which can be used to collect spectral data is generated by the PACGRAM (Psuedo Auto Correction Chromatogram) which has been described in section 4.3.2. The response in the chromatogram is obviously dependent on the difference between the spectrum obtained and the background spectrum. So in the case of the FAME's at nanogram levels, where only the strongest bands will be observed at low intensities, the difference between the spectra of the FAME's and the background can be smaller than the response produced by noisy carrier gas spectra. Consequently, the response of the FAME spectrum at nanogram levels does not register above the noise in the IR chromatogram.

A program was provided by Perkin Elmer based upon the spectral window chromatogram technique, which as the name implies, uses only a small section of the spectrum to produce the IR chromatogram. In the case of FAME's this region would include the C-H stretching region, which has the highest response. The main drawback of this technique is that the regions of data collection must either be selected manually, or using the FID trigger, and reanalysed after the run has finished, therefore not on the fly. Another problem was that only 64 spectra could be collected and stored in the computer per run. This meant that for a long run the spectral capacity was insufficient. For example, using the fast mode and the MCT detector, a spectrum takes 0.03 minutes, hence 64 spectra, taken sequentially, would take 1.92 minutes. Typically, a GC run is 15 to 30 minutes, which means that the spectral capacity is insufficient. To overcome this problem just the region of interest was selected.

4.4.5 Vapour phase spectra of various FAME's obtained using the narrow bore capillary GC-FTIR system

The vapour phase spectra of the FAME's listed below were obtained using the MCT detector and the conditions described in section 4.4.2. These spectra were compared against each other and their corresponding liquid phase spectra to assess the spectral differences.

Methyl elaidate (figure 4.4.5.1)  Methyl linoleate (figure 4.4.5.4)
Methyl oleate (figure 4.4.5.2)  Methyl linolenate (figure 4.4.5.5)
Methyl stearate (figure 4.4.5.3)  Methyl gamma linolenate (figure 4.4.5.6)
The vapour phase spectra obtained resemble closely the corresponding liquid phase spectra, except for the spectrum of methyl linolenate. All of the corresponding vapour and liquid phase spectra showed differences in the relative intensities of absorption bands. This is best illustrated by figure 4.4.5.7 which shows the liquid and vapour phase spectra of methyl elaidate superimposed. Both the carbonyl stretch and trans =C-H deformation bands have significantly lower intensities (relative to the asymmetrical C-H stretching band) in the vapour phase than in the liquid phase spectrum. This is attributed to the lower resolution (8 cm\(^{-1}\) compared with 2 cm\(^{-1}\)) used to take the vapour phase spectrum, which has the effect of reducing and broadening bands. Also apparent in figure 4.4.5.7 is the higher frequency of the carbonyl stretching band in the vapour phase spectrum, which is probably due to reduced hydrogen bonding.

A distinctive feature observed in the vapour phase cis-FAME’s spectra, was the presence of the =C-H stretching band at \(\approx 3005\) cm\(^{-1}\). This band increases in intensity, relative to the other aliphatic C-H stretching bands, with increase in the number of cis double bonds present, as observed in the liquid phase spectra. Methyl linolenate was apparently the only exception. The author was able to ascertain that the spectrum was that of a degradation product of methyl linolenate because the cis =C-H stretching band was absent. Furthermore, the intensity ratios of the major bands observed did not correspond with those of the other FAME’s studied. An investigation based on the above observations was conducted to establish whether the relationship between the intensity ratio of C-H/C=O bands and the number of methylenes in the chain length of various FAME’s, could be used to identify chain length and degree of unsaturation of unknown FAME’s. Details of this study and the influence of temperature and resolution upon these band intensity ratios can be found in section 4.4.8.2, 4.4.8.3 and 4.4.8.4. The results obtained in this section were considered promising because they showed that saturated and unsaturated FAME’s can be distinguished from their vapour phase spectra.

4.4.6 GC-FTIR study of fat N88 using a narrow bore capillary column (0.32mm i.d.)

The effects of different conditions on the IR chromatogram and IR spectra obtained with a narrow bore capillary column using a FAME solution of fat N88 are summarised below:
A. Resolution of IR spectra: When a resolution of 2 cm\(^{-1}\) was used to obtain the IR spectra, the spectral data produced were found to be so noisy that only bands of major intensity could be distinguished. Decreasing the resolution reduced the noise dramatically, however it also reduced the amount of information obtainable from the spectra. For example, when a resolution of 16 cm\(^{-1}\) was used, the cis =C-H stretch of methyl oleate was unresolved from that of the aliphatic C-H stretching vibrations. A good compromise between signal to noise ratio and resolution was obtained using a resolution of 8 cm\(^{-1}\).

B. Number of background scans accumulated: The number of background scans accumulated (co-added) was found to have no effect upon either the IR chromatogram or the spectra produced. Bleeding of the CP Sil88 polar column was found to be a problem because it increased with temperature, thus changing the background spectra and dramatically reducing the quality of the spectra obtained. To overcome this problem isocratic runs were performed using this column, thus maintaining the same level of column bleed and a constant background. In some instances where the spectral quality was impaired, due to poor background subtraction, a spectrum taken either before or after the eluted sample was subtracted using the difference OBEY program.

C. Gain: Increasing the gain (amplification of signal received from the MCT detector) was found to increase both the observed response on the eluted peaks on the IR chromatogram and the intensity of the bands in the IR spectra. However, the noise in both the IR chromatogram and spectra increased. An acceptable increase of response without excessive noise was obtained by increasing the gain setting from one to four.

D. Temperature of light pipe/transferline: Increasing the temperature of the light pipe/transferline was found to reduce the extent to which the eluted sample peaks broaden in the light pipe and transferline, thus increasing the response in the IR spectrum and chromatogram.

E. Effect of light pipe volume: The appreciable volume (570 µl) of the light pipe is clearly a very important factor, as the volume of an eluted FAME has to be sufficient to fill the light pipe, to ensure that only its spectrum is recorded. Otherwise spectra of two or more FAME's occupying the light pipe simultaneously will be seen. In this section a typical hydrogenated fat (N88, see table 4.4.6.1 and figures 3.4.5.2.1) was used to assess whether the vapour phase spectra of individual FAME's could be obtained using the standard capillary GC conditions.
The preliminary GC-FTIR studies, using the standard capillary GC conditions (see table 4.4.6.2), produced no response in the IR chromatogram or any IR spectra. It was necessary to drastically reduce the split ratio to approximately 20:1 before any spectra could be obtained (see Table 4.4.6.3, for further details). Figure 4.4.6.1 and 4.4.6.2 show both the FID and IR chromatograms of a 0.4 μl and 1 μl injections of the sample. Unfortunately, these conditions led to overloading of the column which in turn resulted in very poor resolution of the FAME's in both the chromatograms. Only one significant peak was observed above the noise in either of the IR chromatograms for the reasons outlined in section 4.4.4. However, a considerable number of FAME spectra were obtained with no response in the IR chromatograms, the position of which are indicated on figures 4.4.6.1 and 4.4.6.2. All of these FAME spectra had recognisable bands corresponding to the C-H stretch and C=O stretch. The other regions of the IR spectra were not always distinguishable from the noise, except for the spectrum of the main peak produced with the 1 μl injection (figure 4.4.6.3), where the cis =C-H stretching vibration and the trans band at 969 cm⁻¹ are clearly distinguishable. This implies that the cis and trans isomers of 18:1Δ were either unresolved or co-eluting in the light pipe. It is evident from the FID chromatogram that this is can be attributed to a loss in resolution due to overload of the column.

The following calculations show that the requirement of sufficient volume to fill the light pipe was not satisfied under the conditions of our preliminary studies: In the case of the standard GC conditions (table 4.4.6.2), data generated from the 0.4 μl injection (reported in table 4.4.6.1) was used in conjunction with equation 4.6.6.1, which estimates the volume of a FAME eluted from the column.

**Equation 4.6.6.1; used to determine the volume of an eluent.**

\[ V = \pi r^2 L \]

**Here**

- \( V \) = volume sample of FAME from capillary column
- \( r \) = radius of capillary column in centimeters
- \( L \) = WHH x u
- \( WHH \) = width of peak at half height (seconds)
- \( u \) = linear gas velocity (cm/sec)
Table 4.4.6.1: Composition of fat N88 established by standard capillary GC method described in section 2.3.4. ? = unidentified.

<table>
<thead>
<tr>
<th>FAME</th>
<th>PERCENTAGE</th>
<th>RETENTION TIME (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:0</td>
<td>&lt; 0.5</td>
<td>3.6</td>
</tr>
<tr>
<td>12:0</td>
<td>&lt; 0.5</td>
<td>3.9</td>
</tr>
<tr>
<td>14:0</td>
<td>&lt; 0.5</td>
<td>4.5</td>
</tr>
<tr>
<td>15:0</td>
<td>&lt; 0.5</td>
<td>4.8</td>
</tr>
<tr>
<td>16:0</td>
<td>8.8</td>
<td>5.4</td>
</tr>
<tr>
<td>16:1t</td>
<td>&lt; 0.5</td>
<td>5.6</td>
</tr>
<tr>
<td>16:1c</td>
<td>&lt; 0.5</td>
<td>7.0</td>
</tr>
<tr>
<td>17:0</td>
<td>&lt; 0.5</td>
<td>6.0</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>6.5</td>
</tr>
<tr>
<td>18:0</td>
<td>12.5</td>
<td>6.9</td>
</tr>
<tr>
<td>18:1t</td>
<td>32.8</td>
<td>7.4</td>
</tr>
<tr>
<td>18:1c</td>
<td>37.3</td>
<td>7.7</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>7.8</td>
</tr>
<tr>
<td>?</td>
<td>0.8</td>
<td>7.83</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>7.9</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>8.1</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>8.2</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>8.3</td>
</tr>
<tr>
<td>18:2tt</td>
<td>0.6</td>
<td>8.4</td>
</tr>
<tr>
<td>18:2ct</td>
<td>0.6</td>
<td>8.6</td>
</tr>
<tr>
<td>18:2tc</td>
<td>0.5</td>
<td>8.8</td>
</tr>
<tr>
<td>18:2cc</td>
<td>&lt; 0.5</td>
<td>9.0</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>9.2</td>
</tr>
<tr>
<td>20:0</td>
<td>0.5</td>
<td>9.3</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>9.8</td>
</tr>
<tr>
<td>20:1t</td>
<td>&lt; 0.5</td>
<td>10.1</td>
</tr>
<tr>
<td>20:1c</td>
<td>0.6</td>
<td>10.5</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>11.2</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>11.5</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>12.2</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>13.3</td>
</tr>
<tr>
<td>22:1t</td>
<td>&lt; 0.5</td>
<td>14.5</td>
</tr>
<tr>
<td>22:1c</td>
<td>&lt; 0.5</td>
<td>15.1</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>16.2</td>
</tr>
<tr>
<td>?</td>
<td>&lt; 0.5</td>
<td>17.7</td>
</tr>
<tr>
<td>24:0</td>
<td>&lt; 0.5</td>
<td>19.9</td>
</tr>
</tbody>
</table>
Table 4.4.6.2; Details of capillary GC conditions used to generate data in Table 4.4.6.1.

<table>
<thead>
<tr>
<th>GC</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oven</td>
<td>Carlo Erba HRGC 5399 mega series</td>
</tr>
<tr>
<td>Temperature controller</td>
<td>MFC 500</td>
</tr>
<tr>
<td>Detector amplifier</td>
<td>E1450</td>
</tr>
<tr>
<td>Integrator</td>
<td>Perkin Elmer sigma 10</td>
</tr>
<tr>
<td>Top vent flow</td>
<td>2.8 ml/min</td>
</tr>
<tr>
<td>Bottom vent flow</td>
<td>125 ml/min</td>
</tr>
<tr>
<td>FID flow</td>
<td>0.98 ml/min</td>
</tr>
<tr>
<td>Column temp.</td>
<td>195 °C, injector/detector temp. = 250 °C</td>
</tr>
<tr>
<td>% injected entering column based on column split ratios</td>
<td>0.76%</td>
</tr>
<tr>
<td>Linear gas velocity based on injection of methane</td>
<td>26 cm/sec</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Column</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>column</td>
<td>CP Si188, length = 50 m, film thickness= 0.4 microns</td>
</tr>
<tr>
<td>i.d.</td>
<td>0.32 mm, carrier gas H₂</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Injection technique</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Empty needle/ cold needle</td>
<td>sample withdrawn into the barrel and quickly injected once within instrument. Empty liner used in injection port. 0.4 ul injections were made.</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl esters</td>
<td>dissolved in CCl₄ - 0.2 gram/5 ml; total quantity of sample reaching column based on split ratios = 121.6 ng</td>
</tr>
</tbody>
</table>
Table 4.4.6.3; GC-FTIR conditions used to study FAME solution of fat N88

**Column**
- CP Sil88, liquid film thickness = 0.4 microns, i.d. = 0.32 mm
- length = 50 m, carrier gas H₂

**GC-FTIR**

**GC:**
- Bottom split flow = 27.3 ml/min
- Top split flow = 1.8 ml/min
- IR vent flow = 1.2 ml/min
- FID vent flow = 2.13 ml/min

% of sample injected reaching the IR light pipe 3.7%

Linear gas velocity calculated by injecting methane = 32 cm/min

- Temperature of column = 195 °C
- Temperature of injector/detector = 250 °C

**FTIR:**
- Light pipe/transfer line temperature = 200 °C
- resolution 8 cm⁻¹/ gain = 4/ no make-up gas
- data acquisition sensitivity = 5 units
- no. of co-added background spectra = 4

**Sample**
- Methyl ester dissolved in CCl₄ - 0.2 grams/5 ml. Total quantity of sample reaching light pipe
  - for 0.4 μl injection = 92 ng
  - for 1.0 μl injection = 1480 ng
Table 4.4.6.4 shows that the estimated quantities of the four main components entering the capillary column are between 10.7 ng and 45.5 ng, based on the quantity injected and the split ratios. These quantities are consistent with the sample capacity of capillary columns reported in the literature, viz. 90 ng per sample for a 0.32 mm i.d. column with a 0.5 μm film thickness (Sandra, 1985; Herres, 1987). Of course, this value will vary with the nature of the liquid phase but will remain of the same order of magnitude. It is therefore not surprising that the column used was excessively overloaded when 1070 ng of sample had to be used in order to produce a recognisable spectrum. Table 4.4.6.4 includes the estimated volume of methyl stearate (76 μl), which is one of the major components. It is apparent that several FAME's will occupy the light pipe (570 μl) simultaneously, and their combined spectra recorded. In the case of minor components, occupying smaller volumes, the situation regarding the simultaneous occupation of the light pipe would clearly be worse.

Table 4.4.6.4; Quantities of the four main FAME's in the FAME solution of fat N88 reaching the column under the standard operating conditions of the capillary GC method, based on the data from tables 4.4.6.2 and 4.4.6.1.

<table>
<thead>
<tr>
<th>FAME</th>
<th>PERCENTAGE</th>
<th>QUANTITY (ng)</th>
<th>WHH (seconds)</th>
<th>VOLUME (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>8.8</td>
<td>10.7</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18:0</td>
<td>12.5</td>
<td>15.2</td>
<td>3.6</td>
<td>76</td>
</tr>
<tr>
<td>18:1t</td>
<td>32.8</td>
<td>39.9</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>18:1c</td>
<td>37.4</td>
<td>45.5</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

In a similar fashion Tables 4.4.6.5 and 4.4.6.6 show the quantities of the four main FAME's present in the sample of N88 reaching the light pipe and their respective estimated volumes for the 0.4 μl and 1 μl injections, based on the GC-FTIR conditions in Table 4.4.6.3. These results confirm that the light pipe dimensions are too large, even in the case of the 1 μl injection, where the 18:1Δt and 18:1Δc are unresolved. Their combined volume of 309 μl is insufficient to fill the light pipe. Perkin Elmer have recognised this problem and have changed their light pipe dimensions to a smaller volume (300 μl) in their newer GC-FTIR system. Clearly an even smaller volume is desirable when narrower bore capillary column are used.
Table 4.4.6.5; Quantities and volumes of the four main FAME's in FAME solution of fat N88 (0.4 μl injection - total quantity of N88 reaching light pipe = 592 ng) reaching the light pipe in the GC-FTIR system, based on the data from table 4.4.6.3.

<table>
<thead>
<tr>
<th>FAME</th>
<th>PERCENTAGE (%)</th>
<th>QUANTITY (ng)</th>
<th>WHH (seconds)</th>
<th>VOLUME (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>8.8</td>
<td>52.0</td>
<td>3.0</td>
<td>77</td>
</tr>
<tr>
<td>18:0</td>
<td>12.5</td>
<td>74.0</td>
<td>4.5</td>
<td>116</td>
</tr>
<tr>
<td>18:1t/1c</td>
<td>70.2</td>
<td>414.4</td>
<td>10.5</td>
<td>270</td>
</tr>
</tbody>
</table>

Abbreviations used in table 4.4.6.5, PERCENTAGE = % of each FAME in mixture; Quantity = quantity of each FAME reaching the light pipe; VOLUME = volume of each FAME eluted from the column.

Table 4.4.6.6; Quantities/volumes of the four main FAME's in FAME solution of fat N88 (1 μl injection - total quantity of fat N88 reaching light pipe = 1480 ng) reaching the light pipe in the GC-FTIR system, based on the data from table 4.4.6.3. * = component was partially unresolved.

<table>
<thead>
<tr>
<th>FAME</th>
<th>PERCENTAGE (%)</th>
<th>QUANTITY (ng)</th>
<th>WHH (seconds)</th>
<th>VOLUME (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>16:0</td>
<td>8.8</td>
<td>130.0</td>
<td>6.0</td>
<td>154</td>
</tr>
<tr>
<td>18:0</td>
<td>12.5</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>18:1t/1c</td>
<td>70.2</td>
<td>1036.0</td>
<td>12.0</td>
<td>309</td>
</tr>
</tbody>
</table>
Three interlinked fundamental problems became apparent with the GC-FTIR system:

a) Sensitivity or quantity of sample needed to produce a recognisable spectrum on the GC-FTIR system.

b) Dimensions of the light pipe which were too large for the low volumes eluted from a 0.32 mm i.d. capillary column.

c) The sample capacity of column was insufficient to allow a recognisable spectrum to be obtained without seriously overloading the column and reducing its efficiency.

The most obvious means of improving the situation was to use a wide bore column with a thicker film (e.g. 0.53 i.d. with 1 micron film thickness column), thus allowing a larger volume and greater quantity of each component to reach the light pipe. For example, 1 and 5 micron films are reported to have 200 to 500 ng and 1000 to 1500 ng sample capacities, respectively, with peak volumes between half height greater than 300 µl, but this is dependent upon the conditions used. Columns of this type have reduced resolving power and hence tend to elute FAME's according to carbon number and number of double bonds. No separation is obtained between cis and trans isomers. This could, however, be turned into an advantage as the separation of these isomers would not be necessary if a spectrum with sufficiently high signal to noise ratio could be obtained. Quantitation of the cis/trans content could then be carried out by one or more of the IR techniques described in Chapter 3.

4.4.7 GC-FTIR investigations with wide bore column (0.5 mm i.d./1 micron film thickness/25 m)

The wide bore column chosen for these GC-FTIR investigations was a BP 20 column (i.d. 0.5 mm, 25 m length and a film thickness of 1.0 µm), which is equivalent to a polar carbowax 20M. Studies using a thicker film CP Sil88 liquid phase and wider bore would have been preferred, but were not possible because it is necessary to bond the liquid phase to the column to achieve film thicknesses greater than 0.4 microns, and this cannot be achieved with the CP Sil88 liquid phase.
4.4.7.1 Influence of peak broadening upon quantity of eluent in the light pipe

The studies conducted in this section attempt to establish the minimum concentration of FAME in the light pipe required to produce a spectrum in which the C-H and C=O stretching bands can be distinguished from the noise, and furthermore produce a response in the IR chromatogram.

Various volumes of a solution containing five saturated FAME's (methyl laurate - C12; methyl myristate - C14; methyl palmitate - C16; methyl stearate - C18; methyl eicosanoate - C20) were injected into the GC-FTIR system operated under the isothermal conditions described in Table 4.4.7.1. Equation 4.4.6.1 was used to establish the eluent volume between width of peak at half height (WHH) for each of the FAME's. To simplify the task of establishing the concentration of each FAME, it was assumed that all of the sample occupied the peak volume between WHH.

Table 4.4.7.2 shows the various details required to establish the concentration of each FAME occupying the light pipe for different injection volumes and whether an IR spectrum/chromatogram response was obtained. For example, 328 ng of methyl laurate reached the light pipe when a 0.4 \mu l injection was made. The quantity of sample present in the light pipe when the spectrum was taken was 328 ng because the volume of the eluent between WHH (369 \mu l) was less than that of the light pipe (570 \mu l). In the case of methyl myristate (0.4\mu l injection), the eluent volume between WHH (785 \mu l) exceeds that of the light pipe and the concentration of the FAME eluted (0.42 ng/\mu l) is used to calculate the maximum quantity of sample occupying the light pipe at any one time (238 ng).

From these results it is apparent that there is considerable peak broadening with increase in retention time, as expected. This has significant effects upon the concentration of the sample occupying the light pipe and hence the quality of the IR spectrum or the IR chromatogram response. It can be concluded from these results that the quantity of sample occupying the light pipe must be greater than 200 ng to produce an IR response in the chromatogram. It should be noted that the quantity of FAME referred to is not the same as the quantity required to produce a spectrum in which bands due to cis and trans FAME's can be differentiated from noise.

Tailing at the front of the peaks in the FID chromatograms, which is an indicator of sample overload, was observed for methyl eicosanoate in the 0.5 \mu l injection and for methyl stearate and methyl eicosanoate in the 0.6 \mu l injection. This tailing clearly decreases the concentration of the samples to a greater extent than that established from equation 4.4.6.1, and the author is investigating equations which take this into account. Increasing the concentration of the FAME's
can be achieved by increasing the temperature, thereby decreasing the WHH of a peak. However, this also decreases the retention time of FAME's and reduces their resolution. To overcome this problem, temperature programming may be applied, but this has certain drawbacks due to an increase in column bleed, thus changing the background spectrum. For this reason all the GC-FTIR experiments were conducted isothermally.

Table 4.4.7.1; GC-FTIR conditions used to study influence peak broadening upon quantity of sample reaching light pipe.

| Column | BP 20, liquid film thickness = 1 micron, i.d. = 0.5mm  
length = 25 m, carrier gas H2 |
|--------|--------------------------------------------------|
| GC-FTIR| GC:  
Bottom split flow = 131.4 ml/min  
Top split flow = 2.0 ml/min  
IR vent flow = 5.7 ml/min  
FID vent flow = 1.0 ml/min |
| % of sample injected reaching the IR light pipe | 4.1 % |
| Linear gas velocity calculated by injecting methane | 40 cm/min |
| Temperature of column | 155 °C |
| Temperature of injector/detector | 275 °C |
| FTIR:  
Light pipe/transfer line temperature | 200 °C |
| resolution | 8 cm⁻¹  
gain | 4  
no make-up gas |
| data acquisition sensitivity on IR chromatogram | 10 units |
| no. of co-added background spectra | 4 |
| Sample | Concentration of each FAME dissolved in CO2 = 0.1 grams/5 ml  
FAME's present C12, C14, C16, C18 and C20. |
Table 4.4.7.1.2; Influence of peak broadening and quantity injected of various saturated FAME's upon the IR chromatogram response.

<table>
<thead>
<tr>
<th>vol.</th>
<th>FAME</th>
<th>RT</th>
<th>WHH</th>
<th>peak vol.</th>
<th>Quant.</th>
<th>conc.</th>
<th>L-P</th>
<th>IR</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.3</td>
<td>ML</td>
<td>264</td>
<td>4.7</td>
<td>367</td>
<td>246</td>
<td>0.67</td>
<td>246</td>
<td>YES</td>
</tr>
<tr>
<td>0.3</td>
<td>MM</td>
<td>525</td>
<td>9.3</td>
<td>793</td>
<td>246</td>
<td>0.34</td>
<td>192</td>
<td>NO</td>
</tr>
<tr>
<td>0.3</td>
<td>MP</td>
<td>1115</td>
<td>18.6</td>
<td>1461</td>
<td>246</td>
<td>0.17</td>
<td>96</td>
<td>NO</td>
</tr>
<tr>
<td>0.3</td>
<td>MS</td>
<td>2433</td>
<td>46.5</td>
<td>3652</td>
<td>246</td>
<td>0.07</td>
<td>36</td>
<td>NO</td>
</tr>
<tr>
<td>0.3</td>
<td>ME</td>
<td>5401</td>
<td>110.4</td>
<td>8670</td>
<td>246</td>
<td>0.03</td>
<td>16</td>
<td>NO</td>
</tr>
<tr>
<td>0.4</td>
<td>ML</td>
<td>264</td>
<td>4.7</td>
<td>369</td>
<td>328</td>
<td>0.89</td>
<td>328</td>
<td>YES</td>
</tr>
<tr>
<td>0.4</td>
<td>MM</td>
<td>531</td>
<td>10.0</td>
<td>785</td>
<td>328</td>
<td>0.42</td>
<td>238</td>
<td>YES</td>
</tr>
<tr>
<td>0.4</td>
<td>MP</td>
<td>1129</td>
<td>21.4</td>
<td>1681</td>
<td>328</td>
<td>0.20</td>
<td>111</td>
<td>NO</td>
</tr>
<tr>
<td>0.4</td>
<td>MS</td>
<td>2458</td>
<td>45.3</td>
<td>3558</td>
<td>328</td>
<td>0.09</td>
<td>52</td>
<td>NO</td>
</tr>
<tr>
<td>0.5</td>
<td>ML</td>
<td>267</td>
<td>6.0</td>
<td>471</td>
<td>410</td>
<td>0.87</td>
<td>471</td>
<td>YES</td>
</tr>
<tr>
<td>0.5</td>
<td>MM</td>
<td>532</td>
<td>10.2</td>
<td>805</td>
<td>410</td>
<td>0.51</td>
<td>290</td>
<td>YES</td>
</tr>
<tr>
<td>0.5</td>
<td>MP</td>
<td>1129</td>
<td>21.9</td>
<td>1723</td>
<td>410</td>
<td>0.24</td>
<td>136</td>
<td>NO</td>
</tr>
<tr>
<td>0.5</td>
<td>MS</td>
<td>458</td>
<td>47.1</td>
<td>3699</td>
<td>410</td>
<td>0.11</td>
<td>63</td>
<td>NO</td>
</tr>
<tr>
<td>0.5</td>
<td>ME</td>
<td>5395</td>
<td>102.3</td>
<td>8034</td>
<td>410</td>
<td>0.05</td>
<td>29</td>
<td>NO</td>
</tr>
<tr>
<td>0.6</td>
<td>ML</td>
<td>269</td>
<td>4.9</td>
<td>384</td>
<td>492</td>
<td>1.28</td>
<td>492</td>
<td>YES</td>
</tr>
<tr>
<td>0.6</td>
<td>MM</td>
<td>536</td>
<td>10.0</td>
<td>785</td>
<td>492</td>
<td>0.63</td>
<td>357</td>
<td>YES</td>
</tr>
<tr>
<td>0.6</td>
<td>ME</td>
<td>5474</td>
<td>122.4</td>
<td>9613</td>
<td>492</td>
<td>0.05</td>
<td>29</td>
<td>NO</td>
</tr>
</tbody>
</table>

Where:
- vol. = volume injected (μl)
- RT = retention time (seconds)
- WHH = peak width at half height (seconds)
- peak vol. = peak volume established using equation 4.4.6.1 and data in Tables 4.4.7.1 and 4.4.7.2. (μl)
- Quant. = total quantity of FAME reaching light pipe (ng)
- conc. = concentration of FAME ng/μl ( = Quant./peak vol.)
- L-P = maximum quantity of FAME occupying light pipe (ng)
- IR = whether response is observed in IR chromatogram and IR spectrum is also obtained
4.4.7.2 Optimisation of GC-FTIR system with wide bore capillary column for mixture of five saturated FAME's

The GC-FTIR system with the wide bore capillary column BP 20 described in the previous section was optimised for the same mixture of five saturated FAME's, so that IR spectra and responses could be obtained in the IR chromatogram for all of the FAME's. This was achieved by increasing the quantity of sample injected to 1 µl. Tailing of peaks due to overload of the column was reduced by increasing the temperature to 220 °C. This also had the desired effect of reducing the peak widths and furthermore reducing the retention time of each FAME without compromising the separation. To prevent band broadening in the transferline and light pipe, their temperatures were increased to 225 °C. Further operating details can be found in Table 4.4.7.2.1.

Problems were encountered with both the IR and FID triggers used to start the acquisition of IR spectra. These triggers are activated when the response exceeds a predetermined value in the FID or IR chromatogram. Data acquisition is terminated once the response goes below the selected value. During spectral acquisition the spectra are co-added to reduce noise. In the case of the IR trigger the problem is due to the low response of the FAME's in the IR chromatogram (see section 4.4.4). With the FID trigger, the main problem is the selection of the delay time (Δt) required to allow for the passage of the eluent through the transfer line (this problem is not present in the IR trigger). When the response goes below a selected value, spectral acquisition stops after the same period of time, Δt. If the delay is set too short, no problems will occur for an eluent having a narrow peak because there will be a small delay before most of the eluent enters the light pipe. However, in the case of a broad peak, spectra of the less concentrated tailing shoulder will be obtained. The resultant co-added spectrum will be of poor quality. If the delay time is increased to suit a broad peak, then a narrow peak could be missed. Both triggering modes have the problem that concentration effects of the peak shoulders, or inclusion of background spectra will reduce the quality of the resultant spectrum.

It was found best not to use either of the triggers. Instead the time periods for data acquisition were selected beforehand, and the peak slicing option used. This produces several separate spectra from which the best can be selected (see Table 4.4.7.2.1 under data acquisition). If a particular broad peak is studied and a number of spectra of similar transmittance values and signal to noise value are obtained then they can be co-added to improve the signal to noise ratio. The only drawback with this type of data collection is that a sample must be run on the system to establish the periods of data collection, before any IR spectra are taken.
Table 4.4.7.2.1; GC-FTIR optimum conditions used in study of FAME mixture.

<table>
<thead>
<tr>
<th>Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP 20, liquid film thickness = 1 micron, i.d. = 0.5mm</td>
</tr>
<tr>
<td>length = 25 m, carrier gas H₂</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GC-FTIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC:</td>
</tr>
<tr>
<td>Bottom split flow = 133 ml/min</td>
</tr>
<tr>
<td>Top split flow = 3.3 ml/min</td>
</tr>
<tr>
<td>IR vent flow = 4.4 ml/min</td>
</tr>
<tr>
<td>FID vent flow = 0.85 ml/min</td>
</tr>
</tbody>
</table>

| % of sample injected reaching the IR light pipe 3.1 %                |
| Linear gas velocity calculated by injecting methane = 36 cm/min      |

| Temperature of column = 220 °C                                     |
| Temperature of injector/detector = 275 °C                          |

<p>| FTIR:                                                                  |
| Light pipe/ transfer line temperature = 225 °C                     |
| resolution 8 cm⁻¹, gain = 4/ no make-up gas                         |
| no. of co-added background spectra = 16                              |
| data acquisition - used slice management with the following defined periods; |</p>
<table>
<thead>
<tr>
<th>period</th>
<th>start</th>
<th>stop</th>
<th>option</th>
<th>width</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.59</td>
<td>1.94</td>
<td>slice</td>
<td>0.03</td>
</tr>
<tr>
<td>2</td>
<td>2.04</td>
<td>2.39</td>
<td>slice</td>
<td>0.03</td>
</tr>
<tr>
<td>3</td>
<td>2.87</td>
<td>3.21</td>
<td>slice</td>
<td>0.03</td>
</tr>
<tr>
<td>4</td>
<td>4.34</td>
<td>5.06</td>
<td>slice</td>
<td>0.03</td>
</tr>
<tr>
<td>5</td>
<td>7.04</td>
<td>7.77</td>
<td>slice</td>
<td>0.03</td>
</tr>
</tbody>
</table>

Where; width = period in seconds over which spectrum is taken.

<table>
<thead>
<tr>
<th>Sample</th>
</tr>
</thead>
<tbody>
<tr>
<td>Concentration of each FAME in solution = 0.1 grams/5 ml</td>
</tr>
<tr>
<td>FAME's present C12, C14, C16, C18 and C20.</td>
</tr>
</tbody>
</table>

Table 4.4.7.2.2 shows the peak volumes and quantities in the light pipe for each saturated FAME, achieved using the optimised GC-FTIR conditions. Figure 4.4.7.2.1 shows the IR and FID chromatograms obtained under the optimum conditions. Figure 4.4.7.2.2 shows the best single spectra of the FAME's, and the corresponding co-added spectra. As expected the quality of the spectra deteriorated with the broadening of the peaks. Also of interest was the relative ratios of the C-H and C=O stretching bands in the spectra of this series of saturated FAME's. These band ratios were observed to increased with increase in methylene chainlength. In section 4.4.8 the
feasibility of using those band ratios to distinguish saturated FAME's of different chainlengths was investigated.

Table 4.4.7.2.2; Information required to establish maximum quantity of FAME occupying light pipe at one time. See Table 4.4.7.1.2 for explanation of RT, WHH, etc..

<table>
<thead>
<tr>
<th>vol.</th>
<th>FAME</th>
<th>RT</th>
<th>WHH</th>
<th>peak vol.</th>
<th>Quant.</th>
<th>conc.</th>
<th>L-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>ML</td>
<td>104</td>
<td>2.5</td>
<td>177</td>
<td>620</td>
<td>3.5</td>
<td>620</td>
</tr>
<tr>
<td>1.0</td>
<td>MM</td>
<td>133</td>
<td>3.5</td>
<td>247</td>
<td>620</td>
<td>2.5</td>
<td>620</td>
</tr>
<tr>
<td>1.0</td>
<td>MP</td>
<td>184</td>
<td>5.1</td>
<td>360</td>
<td>620</td>
<td>1.7</td>
<td>620</td>
</tr>
<tr>
<td>1.0</td>
<td>MS</td>
<td>277</td>
<td>7.8</td>
<td>551</td>
<td>620</td>
<td>1.1</td>
<td>620</td>
</tr>
<tr>
<td>1.0</td>
<td>ME</td>
<td>441</td>
<td>15.0</td>
<td>1060</td>
<td>620</td>
<td>0.58</td>
<td>333</td>
</tr>
</tbody>
</table>

4.4.8. Absorbance band ratios

Quantitative work utilising the IR spectra obtained from a GC-FTIR is extremely difficult because many variable factors such as linear gas velocity, temperature of light pipe/transfer line/oven, split ratios and speed of scan all have some influence upon the quantity of sample present in the light pipe at the time the spectrum is taken. This in turn means that quantitation of the FAME can not be made from IR spectra absorbance measurements when the peak volume eluted from the column is greater than the light pipe volume. Peak area measurements from the IR chromatogram could be used, however, the responses of FAME's have been shown to be low and hence often difficult to quantitate due to the noise. On account of these factors the quantitative aspect of the technique was centred on the FID chromatogram, which was shown to have no problems with respect to sensitivity, while the qualitative aspect focussed on the IR spectra obtained.

In section 4.4.5 the spectrum obtained for methyl linolenate was identified as corresponding to a short chain decomposition product due to the relative intensity of the methylene C-H stretching band at 2922 cm⁻¹ compared to that of the carbonyl C=O stretching band (1754 cm⁻¹). Comparison of the vapour phase spectra of the saturated FAME's studied in the previous section showed that the intensity of the methylene C-H stretching band increases with respect to the
carbonyl C=O stretching band with increase in chain length. These observations suggested that
the absorbance intensity ratio of these bands could be used to identify saturated FAME's. To do
this it is necessary to assume that the extinction coefficient of the carbonyl band remains constant
for all FAME's irrespective of the number of methylenes in the chain or the configuration and
number of double bonds. This was found to be true for the solution phase spectra of various
saturated and unsaturated FAME's. Therefore, assuming that this holds true for the vapour phase
spectra, the absorbance of a particular band, relative to that of the carbonyl band, will be
proportional to the molar extinction coefficient of the band. The relative absorbances are referred
to as absorbance band ratios.

The band ratios referred to in the following sub-sections are:

\[
\begin{align*}
C-H/C=O \quad & C-H2/C=O \\
C-H/C-H2 \quad & cis =C-H/C=O \\
trans =C-H/C=O
\end{align*}
\]

Where:

\[
\begin{align*}
C=O & = \text{Absorbance of carbonyl stretch at 1754 cm}^{-1} \\
C-H & = \text{Absorbance of asymmetric methylene carbon hydrogen stretch at 2922 cm}^{-1} \\
C-H2 & = \text{Absorbance of symmetric methylene carbon hydrogen stretch at 2852 cm}^{-1} \\
cis C-H & = \text{Absorbance of cis double bond C-H stretch at 3005 cm}^{-1} \\
trans C-H & = \text{Absorbance of trans double bond C-H deformation band at 969 cm}^{-1}
\end{align*}
\]

The absorbance values were established by drawing a straight baseline and measuring the
absorbance from this to the peak maximum, as shown in figure 4.4.8.1.

4.4.8.1 Assessment of the standard deviation of the band ratios

One of the major problems with the use of band ratios for identification of a FAME is the quality of
the spectrum obtained. This can easily be assessed by a visual examination of the spectrum
which will show if the noise levels are unacceptably high and the errors too great to have
confidence in the band ratio value. However, a more quantitative approach was required. This
was addressed by the NOISE.oy program in section 4.3.3 which establishes the RMS and peak-
to-peak (P-T-P) noise for various regions, from which the standard deviation of the band ratios can

217
Experiments were conducted to assess whether the P-T-P or RMS noise produces the more realistic standard deviation of the band ratio. These involved repeat 1 µl injections of a solution of methyl oleate (1.9917 g/l) into the GC-FTIR system under the same conditions (see Table 4.4.8.1.1) but changing the resolution of the spectrum obtained in each run.

Plots 4.4.8.1.1 and 4.4.8.1.2 show the absorbance ratio of the cis =C-H/C=O bands obtained from the best spectrum produced in each run using different resolutions, with the error bars representing the standard deviation calculated from the P-T-P and RMS noise values, respectively. Surprisingly, the trend observed for the plot with the standard deviation calculated from the RMS noise is the reverse of that produced with the P-T-P noise. According to the RMS plot the noise increases with decrease in resolution, which is clearly wrong. From these results the P-T-P noise was considered the most suitable means of measuring the standard deviation of the band ratio values. An identical trend was observed using the P-T-P noise to calculate the std. dev. of the C-H/C=O band ratio obtained using different resolutions (plot 4.4.8.1.3).

A resolution of 8 cm\(^{-1}\) was considered the most appropriate resolution to use for the vapour phase study of FAME's because it provides a good compromise between reduction in noise and reduction in the value of the ratio. This conclusion agrees with that reached from qualitative visual estimations made in section 4.4.6.

All of the runs conducted in this study at different resolutions produced FID chromatogram peaks with WHH of 5.38 seconds. This corresponds to a peak volume of 423 µl at peak WHH (u = 40 cm/sec) and therefore most of the 4083 ng of sample reaching the light pipe was present in the sample path when the best spectrum was obtained. Thus, there was little or no variation due to quantity of sample reaching the light pipe.

Some experiments were conducted to establish the effect of the GC oven temperature upon the quality of the spectrum of methyl oleate. The column and conditions used were identical to those described in table 4.4.8.1.1, except for the resolution which was kept constant at 8 cm\(^{-1}\) and the oven temperature which was varied for each run as indicated in the plots. 1 µl as well as 5 µl injections of the methyl oleate solution were made. The split ratios on the GC varied with oven temperature and are indicated on Table 4.4.8.1.2, which also includes the peak WHH and the maximum amount of sample present in the light pipe at each temperature.
Table 4.4.8.1.1: GC-FTIR conditions used to acquire IR spectra of methyl oleate using various resolutions.

<table>
<thead>
<tr>
<th>Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>BP 20, liquid film thickness = 1 micron, i.d. = 0.5mm</td>
</tr>
<tr>
<td>length = 25 m, carrier gas H₂</td>
</tr>
</tbody>
</table>

**GC-FTIR**

**GC:**
- Bottom split flow = 95.8 ml/min
- Top split flow = 3.7 ml/min
- IR vent flow = 4.34 ml/min
- FID vent flow = 0.75 ml/min

% of sample injected reaching the IR light pipe 4.1%

Linear gas velocity calculated by injecting methane = 40 cm/min

- Temperature of column = 220 °C
- Temperature of injector/detector = 275 °C

**FTIR:**
- Light pipe/transfer line temperature = 225 °C
- gain = 4/ no make-up gas
- no. of co-added background spectra = 16
- data acquisition - IR trigger (thresh/hold limit set at 10, option peak slice on, with width of 0.03 minutes)

Plots 4.4.8.1.4 and 4.4.8.1.5 show the effect of temperature upon the methyl oleate cis-C-H/C=O and C-H/C=O band ratios, respectively. These plots clearly show that noise or standard deviation of the various absorbance ratios decreases with increase in GC oven temperature. This is due to the narrowing of the FAME peak, consequently increasing the concentration of the sample in the light pipe (see Table 4.4.8.1.2).
Table 4.4.8.1.2; Effects of oven temperature upon quantity of sample present in light pipe.

<table>
<thead>
<tr>
<th>Oven temp.</th>
<th>L-P %</th>
<th>u</th>
<th>WHH</th>
<th>volume</th>
<th>total L-P</th>
<th>Quant. L-P</th>
</tr>
</thead>
<tbody>
<tr>
<td>225</td>
<td>4.1</td>
<td>40</td>
<td>5.58</td>
<td>423</td>
<td>4083</td>
<td>4083</td>
</tr>
<tr>
<td>200</td>
<td>4.0</td>
<td>34</td>
<td>9.3</td>
<td>620</td>
<td>3983</td>
<td>3983</td>
</tr>
<tr>
<td>190</td>
<td>4.1</td>
<td>34</td>
<td>13.9</td>
<td>928</td>
<td>4083</td>
<td>2508</td>
</tr>
<tr>
<td>180</td>
<td>4.2</td>
<td>34</td>
<td>19.8</td>
<td>1321</td>
<td>4183</td>
<td>1805</td>
</tr>
<tr>
<td>170</td>
<td>4.6</td>
<td>34</td>
<td>28</td>
<td>1869</td>
<td>4581</td>
<td>1397</td>
</tr>
<tr>
<td>160</td>
<td>5.1</td>
<td>34</td>
<td>118.6</td>
<td>7918</td>
<td>25394</td>
<td>1828</td>
</tr>
</tbody>
</table>

Where:
- Oven temp. = temperature of oven
- L-P % = percentage of sample injected reaching light pipe
- $u$ = linear gas velocity (cm/sec)
- WHH = peak width at half height (FID chromatogram)
- volume = volume of peak at WHH
- Total L-P = total quantity of sample reaching light pipe
- Quant L-P = quantity of sample present in light pipe when spectrum is taken.

The extent to which the oven temperature can in increased, in order to improve the signal to noise ratio of a FAME spectrum, is governed by two factors. Firstly, the maximum operational temperature of the column or light pipe (approximately 250 °C for both). Secondly, the resolution of two adjacent FAME peaks, which decreases with increase in temperature. The latter generally tends to be the limiting factor, thus a compromise has to be made between resolution and temperature of the GC oven.
4.4.8.2 Band ratio plots of saturated FAME's

Vapour phase spectra of various saturated FAME's were used to produce plots of several ratios of bands (absorbance) versus the number of carbons or methylenes in the corresponding FAME chain. These plots were compared against identical plots produced from the corresponding solution phase spectra in order to establish whether the carbonyl band can be used as a reference band and hence the ratio of certain bands utilised to identify FAME's.

The band ratios studied were C-H/C=O and the C-H2/C=O from the spectra of the saturated FAME's produced under conditions listed in Table 4.4.8.1.1. Table 4.4.8.2.1 summarises the regression data obtained for each of the plots. The correlation coefficients of all the plots were good, except for the C-H/C-H2 plots in both phase, which were not included because the values tended to remain constant irrespective of chain length. Differences may be attributed to the different resolutions used to obtain the solution (2 cm\(^{-1}\)) and vapour (8 cm\(^{-1}\)) phase spectra.

The vapour phase plots of the C-H/C=O and C-H2/C=O band ratios showed identical trends to those of the corresponding solution phase plots. This confirms that the carbonyl band can be used as a reference band in the vapour phase and that the differences between band ratios are sufficient to positively identify saturated FAME's.

Table 4.4.8.2.1; regression data of the solution and vapour phase plots of various band ratios vs. number of carbons or methylenes in FAME chain. The following saturated FAME's were used to generate the plots - 14:0, 16:0, 17:0, 18:0, 20:0 and 22:0.

<table>
<thead>
<tr>
<th>phase</th>
<th>absorbance ratio</th>
<th>plots</th>
<th>correl. coeff.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gradient</td>
<td>Intercept y = 0</td>
</tr>
<tr>
<td>sol.</td>
<td>C-H/C=Oc</td>
<td>0.1398</td>
<td>4.5</td>
</tr>
<tr>
<td>vap.</td>
<td>C-H/C=Oc</td>
<td>0.262</td>
<td>4.6</td>
</tr>
<tr>
<td>sol.</td>
<td>C-H/C=Om</td>
<td>0.1398</td>
<td>2.5</td>
</tr>
<tr>
<td>vap.</td>
<td>C-H/C=Om</td>
<td>0.2620</td>
<td>2.6</td>
</tr>
<tr>
<td>sol.</td>
<td>C-H2/C=Oc</td>
<td>0.082</td>
<td>5</td>
</tr>
<tr>
<td>vap.</td>
<td>C-H2/C=Oc</td>
<td>0.0995</td>
<td>4.4</td>
</tr>
<tr>
<td>sol.</td>
<td>C-H2/C=Om</td>
<td>0.082</td>
<td>3</td>
</tr>
<tr>
<td>vap.</td>
<td>C-H2/C=Om</td>
<td>0.0995</td>
<td>2.4</td>
</tr>
</tbody>
</table>

Abbreviations on following page.
4.4.8.3 Band ratio plots of unsaturated FAME's

The vapour phase spectra of various cis unsaturated FAME's were used to produce plots of ratios of bands versus either the number of carbons, methylenes or cis double bonds in the FAME chain. These plots were generated to assess whether or not appreciable differences in the band ratios occur. The spectra were produced using the conditions and column described in table 4.4.8.1.1. Below is a list of the unsaturated FAME's studied and the codes used to represent these FAME's on the plots.

Table 4.4.8.3.1; list of unsaturated FAME's studied. Where chain x and y refer to the number of methylenes on either chain; CH3-(CH2)y-CH=CH-(CH2)x-C02CH3

<table>
<thead>
<tr>
<th>FAME</th>
<th>code on plot</th>
<th>no. of methylenes in chain x</th>
<th>chain y</th>
</tr>
</thead>
<tbody>
<tr>
<td>14:1Δ9t</td>
<td>14:1t</td>
<td>7</td>
<td>3</td>
</tr>
<tr>
<td>16:1Δ9c</td>
<td>16:1t or 16:1c9</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>18:1Δ6c</td>
<td>18:1c6</td>
<td>4</td>
<td>10</td>
</tr>
<tr>
<td>18:1Δ9c</td>
<td>18:1c or 18:1c9</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>18:2Δ9c12c</td>
<td>18:2c</td>
<td>7</td>
<td>4</td>
</tr>
<tr>
<td>18:4Δ6c9c12c15c</td>
<td>18:4c</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>19:1Δ10c</td>
<td>19:1c</td>
<td>8</td>
<td>7</td>
</tr>
<tr>
<td>20:1Δ8c</td>
<td>20:1c</td>
<td>6</td>
<td>10</td>
</tr>
<tr>
<td>20:3Δ8c11c14c</td>
<td>20:3c</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>20:4Δ7c10c13c16c</td>
<td>20:4c</td>
<td>5</td>
<td>2</td>
</tr>
</tbody>
</table>
Also included on these plots are difference spectra (denoted by diff) obtained by subtraction of the background spectrum. This was done in order to establish whether the signal to noise ratio could be improved.

Summarised below are the conclusions reached from the various plots:

1. Plot 4.4.8.3.1 shows that the cis $\equiv C\cdot H/\equiv C=O$ ratio is constant for FAME's with one cis double bond, irrespective of the chain length of methylenes on either side of the double bond. It should be noted that this may not apply if the double bond is in close proximity to the carbonyl or the terminal methyl group.

2. Plot 4.4.8.3.2 shows that the cis $\equiv C\cdot H/\equiv C=O$ band ratios of 18:2$\Delta 9c12c$ from two different spectra, obtained on different occasions by injecting different quantities of sample into the GC-FTIR system, are constant. The noise of the 4 $\mu l$ injection spectrum is lowest as expected. The difference spectra offered no real advantage with respect of signal to noise.

3. Plot 4.4.8.3.3 shows that the cis $\equiv C\cdot H/\equiv C=O$ band ratio is the same for two different FAME's with four double bonds and different distributions of methylenes on either side of the double bonds.

4. Plot 4.4.8.3.4 shows that the cis $\equiv C\cdot H/\equiv C=O$ ratio increases linearly with increase in number of isolated double bonds in the FAME. This ratio can clearly be used to determine the number of cis isolated double bonds of a FAME in the vapour phase.

5. Plot 4.4.8.3.5 shows C-H/\equiv C=O band ratios of various saturated and both cis/trans unsaturated FAME's plotted versus the number of carbons in the corresponding FAME chain. From this plot various trends can be observed, which are attributed to the reduction of the C-H band intensity (corresponding to the number of methylenes) with increase in the number of double bonds, as represented by the vertical dotted lines. These could be used to predict the C-H/\equiv C=O band ratios of other FAME's. This data combined with GC retention time could contain sufficient information to establish the chain length and number of double bonds of an unidentified FAME. The configuration of the double bonds present could then be determined from the absence or presence of the cis $\equiv C\cdot H$ or trans $\equiv C\cdot H$ bands in the spectrum. For this information to be extracted from the spectra it is necessary to obtain good signal to noise ratios for the bands to be measured. Less useful information can be derived from the cis $\equiv C\cdot H/\equiv C=O$ band ratios of various FAME's versus the number of methylenes as in plot 4.4.8.3.6.
Band ratio plots for methyl oleate/methyl elaidate mixtures produced with the wide bore column

Having overcome the problems associated with the sensitivity issue by using a wide bore column, a new problem was encountered with respect to the lack of resolution of geometrical isomers found with these columns.

The vapour phase spectra of various standard mixtures of methyl oleate and methyl elaidate (total concentration 20g/l in CCl₄) were used to assess whether the cis \( =C-H \) (3005 cm\(^{-1}\) ) and trans \( =C-H \) bands (969 cm\(^{-1}\) ) can be used quantitatively to determine the cis and trans content of co-eluting geometrical isomers from the spectra produced.

Calibration plots were made of various band ratios versus either cis or trans content. The spectra were generated using the column and GC-FTIR system conditions described in Table 4.4.8.4.1. Each sample solution was injected twice, using 1 μl and 5 μl injections. In the case of the 1 μl injections, 1120 ng of methyl oleate and methyl elaidate combined reaches the light pipe, based on the split ratios of the GC on Table 4.4.8.4.1. The peak WHH of these injections varied between 13 and 8.8 seconds, equivalent to peak volumes at WHH of 909 and 620 μl respectively. From these figures the maximum concentrations of eluents present in the light pipe were estimated to be 702 ng and 1020 ng for the peaks, with volumes at WHH of 909 and 620 μl respectively. These differences were attributed to overload of the column, as shown by variable tailing on the leading edge of the chromatogram peak. This overload is accentuated in the chromatograms produced with the 5 μl injections of the mixtures, where 5600 ng of the combined FAME's reached the light pipe, with peak WHH between 16.7 seconds (1180 μl) and 22 seconds (1555 μl). The calculated maximum quantity of sample occupying the light pipe, based on the figures quoted above, was between 2000 ng and 2700 ng for the 5 μl injections of the samples. It should be stressed that these values are likely to be high because of the excessive tailing.

Plot 4.4.8.4.1 represents the relation between the band ratio of the cis \( =C-H/C=O \) and the cis content of the solutions used to produce the spectra (1 μl injections). This plot shows that as the cis content of the solutions increases, the value of the band ratio increases linearly. Unfortunately, the standard deviations of the band ratio values were unacceptably high for accurate quantitation from the spectra, implying that a greater quantity than 620 to 909 ng of the combined FAME's is required in the light pipe at the time the spectrum is obtained. Attempts to reduce the noise by manually subtracting the background spectrum from the sample spectra had little success, as demonstrated by plot 4.4.8.4.5.
Table 4.4.8.4.1: GC-FTIR conditions used to acquire IR spectra of methyl oleate/ methyl elaidate calibration mixtures.

<table>
<thead>
<tr>
<th>Column</th>
<th>BP 20, liquid film thickness = 1 micron, i.d. = 0.5mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>length = 25 m, carrier gas H₂</td>
</tr>
</tbody>
</table>

**GC-FTIR**

**GC:**
- Bottom split flow = 68.9 ml/min
- Top split flow = 4.0 ml/min
- IR vent flow = 4.6 ml/min
- FID vent flow = 1.0 ml/min

% of sample injected reaching the IR light pipe 5.6 %

Linear gas velocity calculated by injecting methane = 36 cm/min

Temperature of column = 200 °C
Temperature of injector/detector = 275 °C

**FTIR:**
- Light pipe/transfer line temperature = 225 °C
- gain = 4/ no make-up gas
- no. of co-added background spectra = 16
- data acquisition - IR trigger (thresh/hold limit set at 10, option peak slice on, with width of 0.03 minutes)
Table 4.4.8.4.2; regression data for the various calibration plots of the methyl oleate/methyl elaidate solution spectra.

<table>
<thead>
<tr>
<th>Plot no.</th>
<th>band ratio</th>
<th>quant.</th>
<th>plots</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>gradient</td>
<td></td>
</tr>
<tr>
<td>4.4.8.4.1</td>
<td>c=C-H/C=O</td>
<td>1 μl</td>
<td>0.00198</td>
<td>0.182</td>
<td>0.956</td>
</tr>
<tr>
<td>4.4.8.4.2</td>
<td>c=C-H/C=O</td>
<td>5 μl</td>
<td>0.00212</td>
<td>0.178</td>
<td>0.991</td>
</tr>
<tr>
<td>4.4.8.4.3</td>
<td>t=C-H/C=O</td>
<td>1 μl</td>
<td>0.00241</td>
<td>0.0071</td>
<td>0.972</td>
</tr>
<tr>
<td>4.4.8.4.4</td>
<td>t=C-H/C=O</td>
<td>5 μl</td>
<td>0.00226</td>
<td>0.0100</td>
<td>0.996</td>
</tr>
</tbody>
</table>

Where; c = cis, t = trans, quant = quantity of solutions injected, inter = intercept of plot (x = 0), corr = correlation coefficient of plot.

Increasing the quantity injected from 1 μl to 5 μl augmented the maximum quantity of the combined FAME's present in the light pipe at the time the best spectra were taken, resulting in a significant reduction in the noise to more acceptable levels (see plot 4.4.8.4.2) and improving the correlation coefficient of the regression data (see table 4.4.8.4.2). Similar trends were observed for the plots of the trans =C-H/C=O band ratios versus the trans content of the methyl oleate/methyl elaidate mixture solutions spectra.

Results obtained in this section suggest that the cis and trans content of co-eluting geometrical isomers can be determined from vapour phase spectra, provided the spectra have a high enough signal to noise ratio. Unfortunately, the sample quantities required in the light pipe to produce a spectrum with sufficiently good signal to noise caused excessive overload of the wide bore column used. One factor not taken into account in this study is the peak homogeneity of the co-eluting isomers. The following section attempts to address this issue.
4.4.8.5 Homogeneity of the co-eluting methyl oleate and methyl elaidate peak

This section describes peak homogeneity investigations carried out on 50/50 methyl oleate/methyl elaidate solution spectra. These were taken at 0.03 minute intervals during the passage of the GC peaks, produced by injecting 1 μl and 5 μl into the GC-FTIR system. The experimental conditions used are shown in Table 4.4.8.4.1. Table 4.4.8.5.1 lists the various band ratio plots used for this purpose.

Identical trends were observed for the band ratio plots of produced from the 1 μl injection. These showed that all the band ratios remained constant, with the same noise level, for the five spectra at the centre of the peak (peak maximum at approximately 6.92 minutes), but differed on the shoulders of the peaks (i.e. <6.86 minutes and >6.98 minutes). This was attributed to lower concentrations of sample in the light pipe and hence increase in noise. The noise tended to be greater in the tailing end of the peak due to the profile of the peak. Similar trends were observed for the peak section spectra produced from the 5 μl injection of the mixture. It was concluded that the peaks showed no appreciable departure from homogeneity.

Table 4.4.8.5.1; List of plots and corresponding band ratios used to study peak homogeneity of co-eluting geometrical isomers.

<table>
<thead>
<tr>
<th>Plot</th>
<th>quant. inj.</th>
<th>band ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4.8.5.1</td>
<td>1 μl</td>
<td>cis =C-H/C=O</td>
</tr>
<tr>
<td>4.4.8.5.2</td>
<td>1 μl</td>
<td>trans =C-H/C=O</td>
</tr>
<tr>
<td>4.4.8.5.3</td>
<td>1 μl</td>
<td>C-H/C=O</td>
</tr>
<tr>
<td>4.4.8.5.4</td>
<td>5 μl</td>
<td>cis =C-H/C=O</td>
</tr>
<tr>
<td>4.4.8.5.5</td>
<td>5 μl</td>
<td>trans =C-H/C=O</td>
</tr>
<tr>
<td>4.4.8.5.6</td>
<td>5 μl</td>
<td>C-H/C=O</td>
</tr>
</tbody>
</table>
4.4.9 Mega bore column/GC-FTIR system

4.4.9.1 Band ratio studies produced by GC-FTIR system utilising a Mega bore column

Two major issues were identified with the GC-FTIR when the wide bore column (BP 20, 0.5 mm i.d., length 25 mm, film thickness 1.0 microns) was used to separate and identify FAME's. The first of these was resolution. The column achieved separation according to chainlength and number of double bonds, but did not separate geometrical isomers. Identification of both saturated and unsaturated classes of FAME's could be ascertained from the various band ratios. Furthermore, these band ratios could be used to quantitate the cis/trans content of co-eluting isomers from the spectra. The second issue is sensitivity. It was necessary to overload the column to obtain the required signal to noise ratio. Although this increased the concentration of the FAME's in the light pipe, it is inefficient because the total volume eluted from the GC column is large, due to the excessive tailing. Two different approaches can be taken to improve sensitivity:

One approach is to increase the sensitivity of the FTIR portion of the GC-FTIR instrument. Work conducted by Griffiths (1977) has shown that for a given peak width there is an optimum light pipe volume which results from a tradeoff between sensitivity and chromatographic resolution. This occurs when $V_{\text{cell}} = V_{1/2}$, where $V_{\text{cell}}$ is the volume of the light pipe and $V_{1/2}$ is the volume of the peak at half height. It is obvious from the experiments conducted using mixtures of saturated FAME's that even during a single GC run the $V_{1/2}$ of the peaks will change. What is usually done in this case is to make $V_{\text{cell}}$ equal to the early peak $V_{1/2}$, and trade off sensitivity towards the higher boiling compounds for GC resolution of the early peaks. However, the GC-FTIR system used was designed for use with packed columns, and subsequently it was nowhere near the optimum for narrow bore capillary columns (<0.5 mm i.d.). The value of $V_{1/2}$ of the peaks (estimated to be 70 µl for 0.32 mm i.d. CP Sil88 column) is considerably smaller than $V_{\text{cell}}$ (570 µl). The obvious solution would be to reduce the size of the light pipe to suit the $V_{1/2}$. Unfortunately this option was not available.

The other approach involves increasing $V_{1/2}$ of the peaks eluted and the sample capacity of the column to compensate for the concentration affect. Two column parameters can be changed to increase the sample capacity, these are film thickness and internal diameter of column. Increases in these two parameters will increase the sample capacity of the column but also impair the separation efficiency.
A Supelcowax 10 column (i.d. 0.75 mm, length 30 m, film thickness 1.0 microns) was selected for the following experiments, because it offered a good compromise between increase in sample capacity and loss in column efficiency.

4.4.9.2 Effects of sample concentration and volume injected into the mega bore capillary GC-FTIR system upon quality of the spectra obtained

In this section the effects of sample concentration and volume injected into the GC-FTIR system upon the V1/2 of a peak and the quality of spectra are reported. Two solutions of methyl oleate in CCl4 were used and are referred to as "dil" (19.917 g/l) and "conc" (39.83 g/l). Various volumes of these solutions were injected into the mega bore capillary GC-FTIR system under the conditions described in table 4.4.9.2.1.

The effects of changing either the concentration or volume of sample solution on the quality of the spectra are shown in the plots listed below. Details of the quantities reaching the light pipe, V1/2 and maximum concentrations of sample in the light pipe are shown in table 4.4.9.2.2.

Plot 4.4.9.2.1; cis =C-H/C=O band ratio versus quantity of "dil" and "conc" solution injected into the GC-FTIR system.

Plot 4.4.9.2.2; C-H/C=O band ratio versus quantity of "dil" and "conc" solution injected into the GC-FTIR system.

Plot 4.4.9.2.1 shows the relationship between the standard deviation of the cis =C-H/C=O band ratio and the volume of "dil" and "conc" methyl oleate solutions injected. As expected the spectra band ratio standard deviation decreased with quantity injected. The noise levels of the "conc" solution injections were generally half that of the corresponding "dil" injection volume. Identical trends were observed for the plots of the C-H/C=O band ratios.

These results suggest that the standard deviation can be reduced by increasing either the concentration of the sample solution or the volume injected, however, the volume that can be injected is limited to 3 μl and 2 μl, for the "dil" and "conc" solutions respectively. Exceeding these values caused overload of the column. Since the 2 μl of the "conc" solution allows the same quantity of sample to reach the light pipe, as the 4 μl injection of the "dil" solution, without
significant peak broadening, it can be concluded that the concentrated solutions improved the chances of producing a narrower peak and hence increased the sensitivity.

Table 4.4.9.2.1; Use of a mega bore column: GC-FTIR conditions used to acquire IR spectra of methyl oleate solutions

<table>
<thead>
<tr>
<th>Column</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supelcowax 10, liquid film thickness = 1 micron,</td>
</tr>
<tr>
<td>i.d. = 0.75 mm, length = 30 m, carrier gas H₂</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>GC-FTIR</th>
</tr>
</thead>
<tbody>
<tr>
<td>GC:</td>
</tr>
<tr>
<td>Bottom split flow = 247.7 ml/min</td>
</tr>
<tr>
<td>Top split flow = 5.8 ml/min</td>
</tr>
<tr>
<td>IR vent flow = 4.2 ml/min</td>
</tr>
<tr>
<td>FID vent flow = 0.67 ml/min</td>
</tr>
</tbody>
</table>

% of sample injected reaching the IR light pipe 1.8%

Linear gas velocity calculated by injecting methane = 16.8 cm/min

Temperature of column = 220 °C
Temperature of injector/detector = 275 °C

IR:
Light pipe/transfer line temperature = 225 °C
gain = 4/ no make-up gas
no. of co-added background spectra = 16
data acquisition - IR trigger (thresh/hold limit set at 75,
option peak slice on, with width of 0.03 minutes)
4.4.9.2.2; Use of a mega bore column: results

<table>
<thead>
<tr>
<th>sol.</th>
<th>vol. inj.</th>
<th>WHH</th>
<th>( V_{1/2} )</th>
<th>total L-P</th>
<th>Quant L-P</th>
<th>tailing of peak</th>
</tr>
</thead>
<tbody>
<tr>
<td>dil</td>
<td>1</td>
<td>7.8</td>
<td>579</td>
<td>320</td>
<td>315</td>
<td>1/1</td>
</tr>
<tr>
<td>conc</td>
<td>1</td>
<td>8.1</td>
<td>601</td>
<td>640</td>
<td>607</td>
<td>1/1</td>
</tr>
<tr>
<td>dil</td>
<td>2</td>
<td>8.6</td>
<td>638</td>
<td>640</td>
<td>570</td>
<td>2.5/2</td>
</tr>
<tr>
<td>conc</td>
<td>2</td>
<td>9.5</td>
<td>707</td>
<td>1280</td>
<td>1031</td>
<td>2/1</td>
</tr>
<tr>
<td>dil</td>
<td>3</td>
<td>9.8</td>
<td>727</td>
<td>980</td>
<td>753</td>
<td>3.5/2</td>
</tr>
<tr>
<td>conc</td>
<td>3</td>
<td>10.5</td>
<td>779</td>
<td>1920</td>
<td>1405</td>
<td>4/1</td>
</tr>
<tr>
<td>dil</td>
<td>4</td>
<td>10.0</td>
<td>742</td>
<td>1280</td>
<td>983</td>
<td>4/1.5</td>
</tr>
</tbody>
</table>

sol. = "dil" or "conc" solution of methyl oleate

inj vol = volume injected into GC-FTIR system (\( \mu l \))

WHH = width at half height (seconds)

\( V_{1/2} \) = peak volume at half height (\( \mu l \))

Total L-P = total quantity of sample reaching light pipe (ng)

Quant L-P = maximum quantity of sample occupying light pipe (ng)

tailing of peak = distance (mm) from front of peak to peak maximum divided by distance from peak maximum to end of peak (overload of column is considered to occur when this ratio exceeds 2)

Although only 320 ng of methyl oleate reached the light pipe when 1 \( \mu l \) of the "dil" solution was injected into the mega bore GC-FTIR system, the quality of the spectrum is comparable with that produced with approximately 700 to 1000 ng of FAME occupying the light pipe when the wide bore (0.5 mm i.d.) capillary column was used. This comes about because \( V_{cell} \) equals \( V_{1/2} \) for the mega bore system. The decision to sacrifice column efficiency for sample capacity, is therefore justified.
4.4.9.3 Calibration plots obtained from mixtures of methyl oleate/methyl elaidate using the mega bore column

According to the literature provided by the manufacturer, the mega bore capillary column (Supelcowax 10, 0.75 mm i.d.) separates FAME’s with different chain lengths and number of double bonds, but not geometrical or positional isomers. These separation characteristics are identical to those observed for the BP 20 wide-bore column. If quantitation of the cis/trans content is required, then it is necessary to establish whether the cis =C-H/C=O and trans =C-H/C=O band ratios of the spectra obtained can be used quantitatively, as described in section 4.4.8.4 for the wide-bore capillary GC-FTIR system.

The vapour phase data for various standard mixtures of methyl oleate/methyl elaidate (these solutions were used in section 4.4.8.4), were obtained by injecting 1 μl and 3 μl of each mixture into the mega bore capillary system described in section 4.4.9.2. The data were used to produce the calibration plots, listed in table 4.4.9.3.1, which were compared with those obtained in section 4.4.8.4 using the wide-bore capillary column. Two solutions of each of the methyl oleate/methyl elaidate mixtures were used in these experiments, varying only in the total concentration of the combined FAME’s but not the percentage. Again these are referred to as "dil" (19.9 g/l) and "conc" (39.8 g/l) solutions.

The regression data obtained for the cis content calibration plots in Table 4.4.9.3.1 show that the correlation coefficient for the wide-bore capillary GC-FTIR system (1 μl injections) was considerably better than that obtained from the mega bore capillary GC-FTIR system (1 μl injections). This result is rather misleading because the signal to noise of the wide-bore system is higher than that obtained with the mega bore system, due to narrower peaks and greater concentrations of eluents occupying the light pipe. It is true to say that the amount of sample present in the light pipe with the wide-bore system (702 to 1020 ng) is greater than the mega bore system (302 to 319 ng), but this was only achieved by overloading the wide-bore column. Volumes of peaks at half height (V_{1/2}) eluted from the wide-bore system were between 702 to 1020 μl for the 1 μl injections, appreciably greater than V_{cell} (570 μl), whereas for the mega bore system V_{1/2} is close to V_{cell}, hence the improvement in sensitivity.
Table 4.4.9.3.1: Regression data of the various calibration plots of the cis =C-H/C=O band ratio versus % cis content obtained using the wide-bore (4.4.8.4.1 & 4.4.8.4.2) and mega bore (4.4.9.3.1 & 4.4.9.3.2) systems.

<table>
<thead>
<tr>
<th>Plot no.</th>
<th>band ratio</th>
<th>quant. inj.</th>
<th>gradient</th>
<th>inter.</th>
<th>corr.</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.4.8.4.1</td>
<td>cis =C-H/C=O</td>
<td>1 µl</td>
<td>0.00198</td>
<td>0.182</td>
<td>0.956</td>
</tr>
<tr>
<td>4.4.8.4.2</td>
<td>cis =C-H/C=O</td>
<td>5 µl</td>
<td>0.00212</td>
<td>0.178</td>
<td>0.991</td>
</tr>
<tr>
<td>4.4.9.3.1</td>
<td>cis =C-H/C=O</td>
<td>1 µl</td>
<td>0.00234</td>
<td>0.177</td>
<td>0.886</td>
</tr>
<tr>
<td>4.4.9.3.2</td>
<td>cis =C-H/C=O</td>
<td>3 µl</td>
<td>0.00215</td>
<td>0.186</td>
<td>0.961</td>
</tr>
</tbody>
</table>

quant inj = quantity injected (µl)
inter = intercept of regression plot (x = 0)
corr = correlation coefficient

Increasing the quantity injected into either the wide-bore or mega bore system significantly improved the signal to noise ratio of the spectra and the correlation coefficients of the calibration plots. However, the larger amount of sample caused overloading of the mega bore column as well as the wide-bore. The accompanying loss of resolution is unacceptable for the determination of cis/trans FAME's in complex mixtures.

Peak homogeneity experiments, similar to those described in section 4.4.8.5, were conducted on the mega bore column using the "dil" (1 µl) and "conc" (3 µl) solutions of the 50:50 of methyl oleate/methyl elaidate mixtures. Plots 4.4.9.3.3 and 4.4.9.3.4 show the cis =C-H/C=O band ratios of the spectra taken during peak slicing. When the greater standard deviations at the front and tail of the peaks are taken into account, it is seen that the GC peak is homogeneous within experimental error.
4.4.9.3.2; Details of the quantities reaching the light pipe, peak volumes at half height, concentration of sample in light pipe and symmetry of peaks observed for various solution of methyl oleate/methyl elaidate mixtures injected into mega bore GC-FTIR system.

<table>
<thead>
<tr>
<th>% cis</th>
<th>sol. inj.</th>
<th>vol</th>
<th>WHH</th>
<th>V₁/₂ L-P</th>
<th>total L-P</th>
<th>Quant of peak</th>
<th>tailing factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>dil</td>
<td>1</td>
<td>7.8</td>
<td>579</td>
<td>320</td>
<td>315</td>
<td>1/1</td>
</tr>
<tr>
<td>100</td>
<td>conc</td>
<td>3</td>
<td>10.5</td>
<td>779</td>
<td>1920</td>
<td>1475</td>
<td>4/1</td>
</tr>
<tr>
<td>95</td>
<td>dil</td>
<td>1</td>
<td>7.7</td>
<td>571</td>
<td>320</td>
<td>319</td>
<td>1/1</td>
</tr>
<tr>
<td>95</td>
<td>conc</td>
<td>3</td>
<td>12.1</td>
<td>898</td>
<td>1920</td>
<td>1219</td>
<td>4/1</td>
</tr>
<tr>
<td>87</td>
<td>dil</td>
<td>1</td>
<td>7.9</td>
<td>587</td>
<td>320</td>
<td>311</td>
<td>1/1</td>
</tr>
<tr>
<td>87</td>
<td>conc</td>
<td>3</td>
<td>10.5</td>
<td>779</td>
<td>1920</td>
<td>1405</td>
<td>4/1</td>
</tr>
<tr>
<td>75</td>
<td>dil</td>
<td>1</td>
<td>7.9</td>
<td>587</td>
<td>320</td>
<td>311</td>
<td>1/1</td>
</tr>
<tr>
<td>75</td>
<td>conc</td>
<td>3</td>
<td>10.7</td>
<td>794</td>
<td>1920</td>
<td>1378</td>
<td>4/1</td>
</tr>
<tr>
<td>62</td>
<td>dil</td>
<td>1</td>
<td>8.1</td>
<td>604</td>
<td>320</td>
<td>302</td>
<td>1/1</td>
</tr>
<tr>
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<td>conc</td>
<td>3</td>
<td>11.4</td>
<td>846</td>
<td>1920</td>
<td>1294</td>
<td>4/1</td>
</tr>
<tr>
<td>49</td>
<td>dil</td>
<td>1</td>
<td>8.1</td>
<td>604</td>
<td>320</td>
<td>302</td>
<td>1/1</td>
</tr>
<tr>
<td>49</td>
<td>conc</td>
<td>3</td>
<td>10.5</td>
<td>777</td>
<td>1920</td>
<td>1408</td>
<td>4/1</td>
</tr>
<tr>
<td>37</td>
<td>dil</td>
<td>1</td>
<td>8.1</td>
<td>604</td>
<td>320</td>
<td>302</td>
<td>1/1</td>
</tr>
<tr>
<td>37</td>
<td>conc</td>
<td>3</td>
<td>10.2</td>
<td>757</td>
<td>1920</td>
<td>1446</td>
<td>4/1</td>
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<td>25</td>
<td>dil</td>
<td>1</td>
<td>8.1</td>
<td>604</td>
<td>320</td>
<td>302</td>
<td>1/1</td>
</tr>
<tr>
<td>25</td>
<td>conc</td>
<td>3</td>
<td>9.8</td>
<td>727</td>
<td>1920</td>
<td>1505</td>
<td>4/1</td>
</tr>
<tr>
<td>12</td>
<td>dil</td>
<td>1</td>
<td>8.1</td>
<td>604</td>
<td>320</td>
<td>302</td>
<td>1/1</td>
</tr>
<tr>
<td>12</td>
<td>conc</td>
<td>3</td>
<td>12.3</td>
<td>915</td>
<td>1920</td>
<td>1196</td>
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</tr>
<tr>
<td>5</td>
<td>dil</td>
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<td>320</td>
<td>302</td>
<td>1/1</td>
</tr>
<tr>
<td>5</td>
<td>conc</td>
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<td>9.3</td>
<td>690</td>
<td>1920</td>
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<tr>
<td>0</td>
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<td>302</td>
<td>1/1</td>
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<td>0</td>
<td>conc</td>
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<td>13.1</td>
<td>975</td>
<td>1920</td>
<td>1123</td>
<td>4/1</td>
</tr>
</tbody>
</table>

Abbreviations are on following page
Abbreviations used in Table 4.4.9.3.2.

sol. = "dil" (19.917 g/l combined weight of FAME's) or "concs" (39.83 g/l combined weight of FAME's) methyl oleate/methyl elaidate mixtures

inj vol = volume injected into GC-FTIR system (µl)

WHH = width at half height (seconds)

V_{1/2} = peak volume at half height (µl)

Total L-P = total quantity of sample reaching light pipe (ng)

Quant L-P = maximum quantity of sample occupying light pipe (ng)

tailing factor = distance (mm) from front of peak to peak maximum divided by distance from peak maximum to end of peak (overload of column is considered when the front of the peak is greater than twice the value of the tail of the peak)

The results obtained in this section indicate that sample capacity of the column can be increased by increasing the diameter of the column, as expected. Furthermore, the peak volumes at half height eluted (without overload) from mega bore system for methyl oleate and methyl elaidate are equivalent to V_{cell}, hence increasing the sensitivity. For example, good signal to noise ratios for the high intensity bands (e.g. C-H, C-H\(_2\) and C=O) on the mega bore system were achieved when ~300 ng of a FAME occupies the light pipe. This compares with quantities >1000 ng for the narrow-bore system (0.32 mm i.d.) and 700 to 1000 ng for the wide-bore system. Both of these latter systems were overloaded.

Good signal to noise ratios for the lower intensity bands, such as the cis=C-H and trans=C-H bands, on the mega bore system were achieved by injecting 3 µl of the "concs" solution, enabling between 1196 and 1400 ng to occupy the light pipe at the time the spectra were taken. Unfortunately, under these conditions column overload occurred, and the consequent loss in resolution is unacceptable when one is studying complex mixtures of components such as hydrogenated fats and oils.
4.5 SUMMARY/CONCLUSIONS

The main objective of the work in this chapter was to assess the potential of the Perkin Elmer GC-FTIR system for the quantitation of the cis and trans fatty acid components of fats and oils. Experiments conducted showed that saturated FAME’s can be identified from C-H/C=O band ratio values obtained from the GC-FTIR vapour phase spectra. The unsaturated FAME’s have very similar spectral features to the saturated FAME’s, however, they can be distinguished by the presence or absence of the cis =C-H stretching band at \( \approx 3005 \text{ cm}^{-1} \) and the trans =C-H deformation band at ca. 969 cm\(^{-1}\). The cis =C-H band showed a linear increase in intensity, relative to the carbonyl band, with increase in the number of isolated cis double bonds. Furthermore, the asymmetric methylene C-H stretching band at \( \approx 2922 \text{ cm}^{-1} \) decreases in intensity, relative to the carbonyl band intensity, with increase in degree of unsaturation. The cis =C-H/C=O band ratio was found to remain constant for FAME’s with the same number of double bonds, irrespective of the number of methylenes, or the distribution of methylenes on either the carbonyl or methyl side chains. This may not apply if the cis double bond is in close proximity to the carbonyl, or adjacent to the terminal methyl group. Although such a comprehensive study was not conducted on the vapour phase spectra of trans FAME’s, identical trends were assumed for the trans =C-H band by analogy with the liquid phase studies described in Chapter 3.

From these observations it was concluded that the vapour phase spectrum of a FAME could yield sufficient information to determine the following:

a. Whether the FAME is saturated or unsaturated.

b. The number of cis or trans isolated double bonds.

c. The chain length of a saturated FAME from the C-H/C=O band ratio. For unsaturated FAME’s, further information is required before the chain length can be deduced.

Our system could not provide information concerning the position of the double bonds. To achieve this, higher resolution spectra having a high signal to noise ratio would be required.

Experiments conducted using the narrow bore column GC-FTIR system, capable of separating geometrical and positional isomers under optimum conditions, failed to produce any spectra of a methylated fat sample unless the column was excessively overloaded. Three interlinked problems were identified with the Perkin Elmer system, namely, lack of sensitivity of IR detector of the GC-FTIR, low sample capacity of column (< 100 ng per FAME), and simultaneous occupation.
of FAME's (resolved on GC) in the light pipe at the time spectra were taken, due to the small volumes eluted (<70 µl) and the large volume of the light pipe (570 µl).

To overcome these problems a wide bore column with a thicker film was incorporated into the GC-FTIR system. This allowed a larger volume and greater quantity of sample of each component to reach the light pipe. From the results conducted on a series of saturated FAME's it was concluded that the quantity of sample occupying the light pipe must be greater than 200 ng to obtain either a spectrum, or a response on the IR chromatogram. However, a greater quantity of sample is needed to produce a spectrum where the distinguishing features corresponding to cis and trans FAME's can be differentiated from the noise. The sample capacity of the wide-bore column approached 500 ng for the lower chain length FAME's but decreased to ca. 400 ng for the longer chain length FAME's. Considerable peak broadening was observed with increase in retention time of FAME, as expected. This had repercussions upon the peak volumes at half height, which varied from 400 µl (for short chain FAME) to 10,000 µl (for longer chain FAME), and hence the concentration of the sample occupying the light pipe. This in turn affects the quality of the spectra. The option of changing the temperature during the run to reduce band broadening, was not considered practicable because increases in column bleed change the background spectrum. Furthermore, the column was operated close to its recommended working temperature. The column was clearly, unsuitable for the analyses of real fats which have a wide range of chainlengths. An additional problem was encountered regarding loss in resolution, allowing FAME's to be separated according to chainlength and degree of unsaturation only. Therefore, positional and geometrical isomer with the same chainlength and number of double bonds remained unresolved. Experiments on a series of mixtures, varying in the composition of geometrical isomers, showed that the cis and trans contents of an eluent could be established from the respective cis =C-H/C=O and trans =C-H/C=O band ratios, provided that the noise level was low enough. This could be achieved only if the column was overloaded.

To overcome the problems with the wide bore capillary column, a mega bore capillary column was tried. This column increased the sample capacity of the system to ~1200 ng for C18 isomers, with a peak volume at half height approximately equal to that of the light pipe. Both these factors increased the quality of the spectra obtained for FAME's with 18 carbons in the carbon chain. The mega bore column resolves FAME's according to chain length and degree of unsaturation, but not positional or geometrical isomers. Similar problems are therefore encountered to those described for the wide bore GC-FTIR system, however attempts to use the cis =C-H/C=O and trans =C-H/C=O band ratios were more successful. Experiments conducted on mixtures of
methyl oleate and methyl elaidate showed that it is possible to obtain spectra of sufficient quality to determine the cis/trans contents without overloading the column.

Although promising results were obtained for the IR quantitation of simple cis/trans isomers, the analysis of more complex mixtures of FAME's was considered less likely to be successful. It is probable that, only the main components, present at the load capacity limit of the column, could be fully identified. Minor components may not be present above the limit required for good quality spectra. Furthermore, the volumes of minor components are liable to be significantly smaller than the light pipe volume. The consequence of this is that several minor components could occupy the light pipe simultaneously, complicating the spectral information obtained. Investigations on real samples would be necessary in order to provide a complete assessment of performance.

Several limitations were found with the GC-FTIR system. The first problem was that only 64 spectra could be collected per run. Perkin Elmer have recognised this deficiency and increased this figure to 300. The second problem concerns the large light pipe volume which has severe implications with regards to sensitivity, and selection of column, affecting both load capacity and resolution efficiency. Griffiths (1977) showed that for a GC-FTIR system with a given chromatographic resolution, the optimum sensitivity is achieved when the volume of the cell \( V_{\text{cell}} \) is approximately equal to the volume of the peak at half height \( V_{1/2} \). The mega bore column is most suited to the GC-FTIR system used in the present work, because the volumes are almost the same. Modern GC-FTIR systems have light pipe volumes of about 100 \( \mu \text{L} \) which makes the use of more efficient columns practicable.

The overall assessment of the application of the Perkin Elmer GC-FTIR system to fat and oil analysis, is that identification of different saturated and cis/trans unsaturated FAME's can be achieved, but the performance is severely limited. An alternative approach, is the matrix isolation GC-FTIR technique. Mossoba et al. (1990) successfully used this technique to identify and quantify 18:2\( \Delta 9 \Delta 12 \)t and other trans FAME's in margarines at low levels (e.g. 0.3%\) separated by a narrow bore capillary column. The amounts of each component were as low as 0.9 ng.
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TABLE 1; Saturated fatty acids of general formula CH₃-(CH₂)ₙ-COOH

*Shorthand notation used to describe the structure of the fatty acid (see section 1.1.3)

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<tr>
<th>Systematic name</th>
<th>Common name</th>
<th>Formula</th>
<th>Formula weight</th>
<th>Shorthand designation *</th>
</tr>
</thead>
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<td>Formic</td>
<td>HCOOH</td>
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<td>Propionic</td>
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<td>74.08</td>
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<td>Butyric</td>
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<td>4:0</td>
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TABLE 2: Monoenoic fatty acids of general formula - CH₃-(CH₂)ₗ·CH=CH-(CH₂)ₘ·COOH

* Shorthand notation used to describe the structure of the fatty acid (see section 1.1.3)

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<th>Systematic name</th>
<th>Common name</th>
<th>Formula</th>
<th>Formular weight</th>
<th>Shorthand designation*</th>
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<td>C₁₉H₃₆O₂</td>
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<td>19:1Δ₁₂t</td>
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TABLE 3: Poly-unsaturated fatty acids of general formula  
\[ \text{CH}_3(\text{CH}_2)_M(\text{CH}=	ext{CH-CH}_2)_X(\text{CH}_2)_N\text{COOH} \]

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<th>Trivial name</th>
<th>Shorthand designation</th>
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</thead>
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<td>gamma-linolenic</td>
<td>18:3\Delta6c9c12c</td>
</tr>
<tr>
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<td>homo-gamma-linolenic</td>
<td>20:3\Delta8c11c14c</td>
</tr>
<tr>
<td>5,8,11,14-eicosapentaenoic</td>
<td>arachidonic</td>
<td>20:4\Delta5c8c11c14c</td>
</tr>
<tr>
<td>4,7,10,13,16-eicosapentaenoic</td>
<td>-</td>
<td>20:5\Delta4c7c10c13c16c</td>
</tr>
<tr>
<td>5,8,11,14,17-eicosapentaenoic</td>
<td>-</td>
<td>20:5\Delta6c8c11c14c17c</td>
</tr>
<tr>
<td>7,10,13,16,19-docosapentaenoic</td>
<td>-</td>
<td>22:5\Delta7c10c13c16c19c</td>
</tr>
<tr>
<td>4,7,10,13,16,19-docosahexaenoic</td>
<td>-</td>
<td>22:6\Delta4c7c10c13c16c19c</td>
</tr>
<tr>
<td>5,8,11-eicosatrienoic,</td>
<td>-</td>
<td>20:3\Delta5c8c11c</td>
</tr>
</tbody>
</table>
APPENDIX II

The following basic and obey programs were written:

TAB.ba:- A basic program which retrieves data from two data files called data.da (concentration values) and data.da (absorbance values). Then produces a table of the concentration and absorbance values. This program is incorporated into several of the obey programs (see page 255).

TAB2.ba:- Same as TAB.ba except it will produce a table of conc. vs. "% content" (see page 255).

COM1.ba:- A basic program used by SIM4.oy to select the best fitting methyl stearate spectrum for the baseline (see page 255).

PLOTC.ba:- A basic program which will retrieve data from the two data files mentioned under TAB.ba and plot each corresponding set of points to produce a plot. It also determines the slope and intercept (see page 256).

AREA.oy:- An obey program which retrieves a set of resultant solute spectra from a floppy disc and determines the area of a selected peak (see page 257).

ABSORBPR.oy:- An obey program which retrieves a set of resultant solute spectra from a floppy disc and determines the peak height absorbance (baseline corrected) according to the procedure described in section 3.3.4 (see page 260).

SUBA3.oy:- This obey program will retrieve a series of solution spectra form a floppy disc and subtract the solution spectrum using a normalisation factor of one. The resulting spectra are stored on a floppy disc (see page 263).

SCANTIME.oy:- This obey program takes spectra at defined time intervals and stores them on a floppy disc (see page 265).

SIM4.oy:- An obey program which uses the spectrum of methyl stearate to simulate a baseline. The program COM1.ba is used to determine the best baseline form a library of methyl stearate spectra stored on the hard disc (see page 266).

NOISE.oy:- This program determines the RMS and P-T-P noise of defined spectral regions as described in section 4.3.3 (see page 270).

CUBICA.oy:- This obey program was used to produce the cubic spline simulation baseline. It uses several programs (see page 274).
$10$ print tab(28);"cono";tab(40);"trans peak absorb"
$40$ open "1", #5,"/usr/ir/data/data.da"
$45$ open "1", #6,"/usr/ir/data/data.da"
$50$ i=1
$60$ if eof ( 5 ) goto 120
$70$ input #5,b,x(i)
$75$ input #6,b,y(i)
$80$ print tab(28);x(i);tab(40);y(i)
$90$ i =i+1
$100$ goto 60
$120$ close #5
$130$ system

Sun Jul 16 20:24:49 1989 /y/tab2.ba Page 1

$10$ rem tab2.ba
$20$ open "i", #4,"/usr/ir/data/data.da"
$30$ input #4, b, c, d
$40$ close #4
$50$ print tab(8);"gradient =";c;"slope =";d
$60$ print tab(8);"weight =";tab(20);"absorbance";tab(35);"% content"
$70$ open "i", #5,"/usr/ir/data/data.da"
$80$ open "i", #6,"/usr/ir/data/data.da"
$90$ i = 1
$100$ if eof ( 5 ) goto 180
$110$ input #5,b,x(i)
$120$ input #6,b,y(i)
$130$ s(i) = (x(i)-d)/c
$140$ w(i) = (x(i)*148.25)/y(i)
$150$ print tab(8);y(i);tab(20);x(i);tab(35);w(i)
$160$ i =i+1
$170$ goto 100
$180$ close #5
$190$ close #6
$200$ system

Sun Jul 16 20:21:41 1989 /y/com1.ba Page 1

$25$ defint i
$7$ dim xy(19,2)
$10$ open "i", #5,"/usr/ir/data/data.da"
$15$ for i=1 to 19
$20$ input #5,b,xy(i,1),xy(i,2)
$22$ print ;xy(i,1);xy(i,2)
$23$ if xy(i,2) < 0 then xy(i,2) = -1 * xy(i,2)
$27$ next i
$29$ close #5
$30$ ymin =xy(1,2)
$35$ xmin =xy(1,1)
$40$ for i = 2 to 19
$50$ if xy(i,2) < ymin then ymin =xy(i,2):xmin =xy(i,1)
$59$ next i
$120$ open "o", #3,"/usr/ir/data/mail.da"
$130$ print #3, 1;xmin
$140$ close #3
$150$ system
$10$ define $l,n,k,m$
$20$ dim $x(200),y(200)$
$25$ np=0
$50$ open "," , " /usr/ir/datts/data/data.da"
$55$ open "," , " /usr/ir/datts/data/data.da"
$60$ s1=0
$62$ s2=0
$63$ s3=0
$70$ i=1
$80$ if eof (8) goto 107
$85$ input # 6,b,y(i)
$90$ input # 6,b,x(i)
$95$ np=np+1
$100$ s1=s1+ x(i)*y(i)
$101$ s2=s2+ x(i)*x(i)
$104$ i=i+1
$105$ goto 80
$107$ v=s1/s2
$108$ for i=1 to np
$109$ dy= y(i) - v*x(i)
$110$ s3= s3 + dy*dy
$115$ next i
$120$ sdy= sqr (s3/(np-1))
$130$ sm= .1*int(sdy/sqr(s2)/v*1000+.5)
$140$ close # 6
$145$ close # 5
$150$ ymax=y(1)
$200$ xmax=x(1)
$250$ if y(i)>ymax then ymax=y(i)
$300$ library "/bin/gr_text"
$310$ call grinit
$311$ lo= 60; lx= 659; ly= 250; lun= 1
$315$ call gropen (&lerr, &lo, &lx, &ly, &lun)
$320$ ko= 15; kt= 235
$330$ call window(&ko, &ko, &kt,, &kt)
$340$ mode= 256* 3
$350$ xinc= xmax/10
$370$ yinc= ymax/10
$373$ ymux= ymax+yinc
$374$ xmux= xmax+xinc
$378$ xmins=0; ymins=0
$380$ call scale(&xmins, &ymins, &xmux, &ymux, &mode)
$390$ nt= 12
$400$ call line(&xmins, &ymins, &xmin, &ymax, &ymux, &mode)
$410$ call line(&xmin, &xmax, &ymins, &ymax, &mode)
$419$ mode=8192+ 256* 3 +65536*8
$430$ cf="t"
$440$ for i=1 to np
$450$ call usymb (&x(i), &y(i), &c$, &mode)
$481$ next i

Sun Mar 26 21:58:16 1989 /y/plotc.ba Page 1

453 ymax =v*xmax
461 mode=mode + 65536* 6
463 call line (&xmin, &ymins, &xmax, &ymax, &mode)
464 $s$ = "gradient = " + str$(v)$
465 $b$ = " $b(y) = " + str$(sdy)$
466 $c$ = " $c = " + str$(sm)$ + " $
467$ mod= 65536* 4 + 256* 3 + 8192
$470$ call symb (&0100, &0250, &0, &mode)
$480$ call symb (&0100, &0250, &0, &mode)
$490$ call symb (&0100, &0210, &0, &mode)
500 call gclose(&lerr, lun)
505 call writergr (plot)
510 system
THIS PROGRAM WILL DETERMINE THE AREA OF ONE PEAK
USING A PRESELECTED BASELINE (N1 TO N2)

A = AREA OF PEAK A (BASE LINE CORRECTED)
N1 to N5 = BASE LINE WAVE NUMBERS

&L10*
gclear
do sclear
output clear \e3
output clear \v6

&L20*
A

N1
N2

&L30*

\v0

* ENTER NAME OF SPECTRAL FILE (spectra should be stored on a floppy disc in
* port x and should be in the form \e@ where \e is any letter, this will
* allow the program to retrieve the spectra sequentially)
&ENTER A1
do sclear
#DO YOU WISH TO VIEW THE SPECTRA ? y OR n
&ENTER A2
&IF A2 = "y" THEN L20
&IF A2 = "n" THEN L30
&L20*
do sclear
* ENTER HIGH WAVE NUMBER OF REGION YOU WISH TO VIEW
&ENTER A3
do sclear
* ENTER LOW WAVE NUMBER OF REGION YOU WISH TO VIEW
&ENTER A4
retrieve \v0 &A1
view \v0 &A3 &A4 a aute
do sclear
axiss

*MOVE CURSOR TO HIGH WAVE NUMBER (N1) AND PRESS "H" TO ENTER WAVE NUMBER
* START OF BASELINE
&cursor \v5
do sclear

*MOVE CURSOR TO PEAK WAVE NUMBER (N2) AND PRESS "H" TO ENTER WAVE NUMBER
* START OF PEAK A
&cursor \v6
&L30 *
do sclear
OUTPUT CLEAR v0
*ENTER VALUE OF HIGH WAVENUMBER (N1) OF BASELINE
&ENTER A5
calc v5 =&A5
do sclear
*ENTER VALUE OF START OF PEAK A WAVENUMBER (N2)
&ENTER A6
calc v6 =&A6
do sclear
&A6 =
*DO YOU WISH TO START AGAIN? y OR n -IF YOU WISH TO FINISH TYPE "end"
*OR TYPE "plot" TO OBTAIN A PLOT OF YOUR RESULTS
&ENTER A10
&IF A10 = "y" THEN L10
&IF A10 = "n" THEN L50
&IF A10 = "end" THEN L100
&IF A10 = "plot" THEN L90
do sclear
&L50 =
do sclear
*DO YOU WISH TO HAVE A HARD COPY OF EACH RESULTANT SPECTRUM? y OR n
&ENTER A17
&IF A17 = "y" THEN L51
&IF A17 = "n" THEN L52
&L51 =
do sclear
*ENTER HIGH WAVENUMBER VALUE (V1) OF SPECTRA WHICH YOU WISH PRINTED
&ENTER A21
do sclear
*ENTER LOW WAVENUMBER VALUE (V2) OF SPECTRA WHICH YOU WISH PRINTED
&ENTER A24
&L52 =
do sclear
*ENTER ANY DETAILS YOU WISH PRINTED
&ENTER A25
do sclear
&A25
do print
do pause 2
do sclear
*ENTER NUMBER OF SPECTRA TO BE ANALYSED
&ENTER A26
calc v26 =&A26
&L60 =
do sclear
retrieve x FB;&A1
area x &v5 &v6
calc v13 = v57
calc v14 = v58
do clear
&L1 =
* PEAK AREA
* NOT BASELINE CORRECTED
* BASELINE CORRECTED
A
do print
  incr A1
  calc v20 = v20 - 1
  &IF A17 = "y" THEN L65
  &IF v20 = 0 THEN L70
  &GOTO L60
  &L60 *
  do sclear
  view x &A21 &A24 a aute
  do sclear
  axis
  sprint
  do pause 75
  do sclear
  &IF v20 = 0 THEN L70
  &GOTO L60
  &L70 *
  &GOTO L40
  &L60 *
  do sclear
  sclear
  &GOTO L999
  &L100 *
  do sclear
  &L999 *
  *END
  do sclear
THIS PROGRAM WILL DETERMINE THE ABSORBANCE (X)
OF A PEAK USING A PRESELECTED BASELINE (N1 TO N3)

N1 = HIGH WAVENUMBER
N2 = PEAK WAVENUMBER
N3 = LOW WAVENUMBER

X = ABSORBANCE OF PEAK (BASE LINE CORRECTED)

N1 to N3 = BASE LINE

THIS PROGRAM IS DIFFERENT FROM ABSORBANCE.oy BECAUSE IT WILL PRINT
THE SPECTRA (BETWEEN THE LIMITS V1 AND V2) AND THE RESULTS --SO MAKE SURE
THE EPSON PRINTER IS CONNECTED AND SWITCHED ON

DO YOU WISH TO VIEW THE SPECTRA? Y OR N

ENTER HIGH WAVENUMBER OF REGION YOU WISH TO VIEW

ENTER LOW WAVENUMBER OF REGION YOU WISH TO VIEW

MOVE CURSOR TO HIGH WAVENUMBER (N1) AND PRESS "H" TO ENTER WAVENUMBER

MOVE CURSOR TO PEAK WAVENUMBER (N2) AND PRESS "H" TO ENTER WAVE NUMBER
do sclear
*MORE CURSOR TO LOW Wavenumber (N3) AND PRESS "H" TO ENTER WAVE NUMBER
&cursor v7
do sclear
gclear
&GOTO L40
&L30 =
do sclear
*OUTPUT CLEAR v0
*ENTER VALUE OF HIGH Wavenumber (N1)
&ENTER A5
calc v5 =&A5
do sclear
*ENTER VALUE OF PEAK Wavenumber (N2)
&ENTER A6
calc v6 =&A6
do sclear
*ENTER VALUE OF LOW Wavenumber (N3)
&ENTER A7
calc v7 =&A7
do sclear
*DO YOU WISH TO START AGAIN? y OR n - IF YOU WISH TO FINISH TYPE "end"
*OR TYPE "PLOT" TO OBTAIN A PLOT OF YOUR RESULTS
&ENTER A9
&IF A9 = "y" THEN L10
&IF A9 = "n" THEN L50
&IF A9 = "end" THEN L100
&IF A9 = "plot" THEN L50
&ENTER A9
&IF A9 = "end" THEN L100
&IF A9 = "plot" THEN L50
&ENTER A9
&IF A9 = "y" THEN L51
&IF A9 = "n" THEN L52
&L51 =
do sclear
*ENTER HIGH Wavenumber VALUE (V1) OF SPECTRA WHICH YOU WISH PRINTED
&ENTER A3
do sclear
*ENTER LOW Wavenumber VALUE (V2) OF SPECTRA WHICH YOU WISH PRINTED
&ENTER A4
&L52 =
do sclear
*ENTER ANY DETAILS YOU WISH PRINTED
&ENTER A16
do sclear
*A16
do print
do pause 2
do sclear
*ENTER NUMBER OF SPECTRA TO BE ANALYSED
&ENTER A6
calc v20 =&A6
idris rm /usr/ir/data/date.de
vsave open new v4:date
AL60 *
do sclear
retrieve x F0 &A1
calo v6 = (x(v5))
calo v9 = (x(v6))
calo v10 = (x(v7))
calo v11 = v6 - v10
calo v12 = v5 - v7
calo v13 = v6 - v7
calo v14 = v11=v13/v12
calo v15 = v14 + v10
calo v16 = v9 - v15
vsave w4:date v16
gclear
do sclear
%A1
N1 = &v5 N2 = &v6 N3 = &v7
* BASELINE ABSORBANCE = &v15
* ABSORBANCE OF PEAK &v6 = &v9
* ABSORBANCE OF PEAK &v6 (BASELINE CORRECTED) = &v16
do print
do pause 10
&incr A1
calo v20 = v20 - 1
&IF A17 = "y" THEN L65
&IF v20 = 0 THEN L70
&GOTO L60
&L65 *
do sclear
view x &A3 &A4 a aut
endo sclear
axis
print
ndo pause 75
ndo sclear
&IF v20 = 0 THEN L70
&GOTO L60
&L70 *
vsave close w4:date
&GOTO L40
&L96 *
do sclear
do clear
* HAVE YOU ENTERED YOUR CONCENTRATION (X) VALUES
* TYPE y OR n AND ENTER
&ENTER A11
&IF A11 = "y" THEN L98
&IF A11 = "n" THEN L95
&L95 *
* ENTER NUMBER OF SOLUTIONS (OR X VALUES)
&ENTER &A8
calo v20 = &A8
do sclear
idris rm /usr/ir/data/data.da
vsave open new w4:data

vsave open new w4:date
AL60 *
calo v12 = 1
* ENTER CONCENTRATION (X VALUES)
&ENTER A15
do sclear
vsave v4:date a15
calo v20=v20-1
&IF v20 = 0 THEN L97
&GOTO L96
do sclear
&L97 *
vsave close w4:data
AL90 *
idris basic -147000 -r /usr/ir/data/tab.ba
do print
do pause 2
do sclear
idris basic -147000 -r /usr/ir/data/plotc.ba
idris fullscreen
&print
do pause 2
&GOTO L99
&L99 *
do sclear
&L99 *
* END
do sclear
SUBA3

THIS COMPUTER PROGRAMME WILL SUBTRACT THE SOLVENT SPECTRA FROM A SET OF SOLUTION SPECTRA STORED ON A FLOPPY DISC IN PORT X AND THEN SAVES THE RESULTING SPECTRA ON A FORMATED DISC IN PORT Y.

NOTE: THE SOLUTION SPECTRA SHOULD BE IN THE FORM XX001 WHERE X IS ANY LETTER. THE SOLVENT SPECTRA MUST HAVE DIFFERENT LETTERS XX TO THAT OF THE SOLUTION SPECTRA.

DO YOU WANT TO VIEW THE SOLVENT SPECTRA AND ONE OF THE SOLUTION SPECTRA?

Y OR N

ENTER A1

IF A1 = "y" THEN L20

IF A1 = "n" THEN L100

L20*

ENTER FILE NAME OF SOLUTION SPECTRA YOU WISH TO VIEW

&ENTER A2

RETRIEVE X F0: &A2

DO CLEAR

ENTER FILE NAME OF SOLVENT SPECTRA YOU WISH TO VIEW

&ENTER A3

RETRIEVE Y F0: &A3

DO CLEAR

ENTER HIGH WAVE NUMBER VALUE OF RANGE YOU WISH TO VIEW

&ENTER A4

DO CLEAR

ENTER LOWER WAVE NUMBER VALUE OF RANGE YOU WISH TO VIEW

&ENTER A5

DO CLEAR

DO YOU WISH TO THE AUTO SPECTRA: y or n?

&ENTER A19

IF A19 = "Y" THEN L21

IF A19 = "N" THEN L22

L21*

&DEF A13 = "auto"

DO CLEAR

GOTO L23

L22*
&def A13 = " "
&L23 *=
*DO YOU WISH TO VIEW THE SPECTRA IN TRANSMISSION OR ABSORBANCE ? t or a
&ENTER A6
do sclear
*DO YOU WISH TO VIEW THE SPECTRA SEPARATELY ? y OR n
&ENTER A7
&IF A7 = "y" THEN L24
&IF A7 = "n" THEN L25
do sclear
&L24 *=
*PRESS ENTER TO VIEW SOLUTION SPECTRA
do pause
gclear
view x &A4 &A5 &A6 &A13
do sclear
axis
do pause
*PRESS ENTER TO VIEW SOLVENT SPECTRA
do pause
do sclear
gclear
view y &A4 &A5 &A6 &A13
axis
do pause
do sclear
&GOTO L26
&L25 =
view x &A4 &A5 &A6 &A13
view y &A4 &A5 &A6 &A13
do sclear
axis
do pause
&L26 =
gclear
do sclear
&L27 *=
*DO YOU WISH TO VIEW THE SPECTRA AGAIN OR RESELECT THE HIGH AND LOW
*WAVENUMBERS ? y OR n
&ENTER A8
&IF A8 = "y" THEN L20
&IF A8 = "n" THEN L110
&L100 *=
do sclear
*ENTER FILE NAME OF SOLVENT SPECTRA YOU WISH TO SUBTRACT FROM THE SOLUTION
*SPECTRA
&ENTER A3
retrieve y F0:&A3
do sclear
&L110 *=
*ENTER FILE NAME OF SPECTRAL FILE WHICH WILL RECEIVE RESULTING SPECTRA
&ENTER A9
do sclear
*ENTER NAME OF SOLUTION SPECTRAL FILE - FILE WILL BE RECALLED SEQUENTUALLY
&ENTER A10
do sclear

*ENTER NUMBER OF SOLUTION SPECTRA
&ENTER a18
calo v12 = &a18
&L120 *=
retrieve x F0:&a10
do sclear
diff y = 1
save = F1:&A9
&incr A10
&incr A9
calo v12 = v12 - 1
&IF v12 = 0 then L150
&GOTO L120
&L150 *=
&L999 *
SCANTIME

THIS PROGRAM TAKES SCANS AT A PRESELECTED
TIME INTERVAL AND SAVES THE SCANS ON A
FLOPPY DISC IN PORT Y (1)

Enter resolution required (2, 4, 8 or 16)
Enter mode resolution
Enter number of scans to be accumulated
Enter time interval
Enter number of spectra required
Enter file name (in the form ab001)
Calculate time
Press enter when you are ready to start

Do pause
Scantime

Do clear

Do clear

Do clear

Do clear

Do clear

Do clear

Do clear

Do clear

Do clear

Do clear

Do clear

Do clear

Do clear

Do clear

Do clear
&L10 *
gclear
output clear a0
output clear v0
= SIM4.oy
*
SIMULATED BASELINE CORRECTION TECHNIQUE
*
= 
do pause
do sclear
*ENTER FILE NAME OF SPECTRUM WHICH CONTAINS PEAK (spectra should be stored
#in port x)
&ENTER A2
do sclear
*ENTER VALUE OF WAVE NUMBER USED TO DETERMINE THE MULTIPLICATION FACTOR
*ENTER A6
calc v1 = &A6
do sclear
*ENTER VALUE OF PEAK WAVE NUMBER
&ENTER A7
calc v2 = &A7
do sclear
&L40 *
*DO YOU WISH TO START AGAIN ? Y OR N (IF YOU WISH TO FINISH TYPE "END"
*OR TYPE "PLOT" TO OBTAIN A PLOT OF YOUR RESULTS
&ENTER A9
&IF A9 = "y" then L10
&IF A9 = "n" then L50
&IF A9 = "end" then L100
&IF A9 = "plot" then L90
do sclear
&L50 *
do sclear
*DO YOU WISH TO HAVE A HARD COPY OF EACH RESULTANT SPECTRUM ? Y or n
&ENTER A17
&IF A17 = "y" THEN L51
&IF A17 = "n" THEN L62
&L51 *
do sclear
*ENTER HIGH WAVE NUMBER (V1) OF SPECTRAL REGION YOU WISH PRINTED
&ENTER A4
do sclear
*ENTER LOWER WAVE NUMBER (V2) OF SPECTRAL REGION YOU WISH PRINTED
&ENTER A5
do sclear
*DO YOU WISH A PRINT OR PLOT
&ENTER A21
&L2 *
do sclear
*ENTER ANY DETAIL YOU WISH PRINTED
&ENTER A16
do sclear
*&A16
do print
do sclear
*ENTER NUMBER OF SPECTRAL TO BE ANALYSED
&ENTER A6
calc v20 =A6
idris rm /usr/ir/data/data.da
vsave open new w4:date
do sclear
AL53 *
:idris rm /usr/ir/data/dail.da
vsave open new w4:dail
retrieve x F0:&A2
calc v23 = 12
&edef A1 ="st001"
calc v16 = 1
&L54 *
retrive y w4:&A1
calc v22 = 1020
calc v3 = (x(v1))
calc v4 = (y(v1))
calc v5 = v3/v4
mult y &v5
calc v6 = (x(v22))
calc v7 = (y(v22))
calc v8 = v6 - v7
&L55 *
vsave w4:dail v16,v8
calc v16 =v16 + 1
&IF v23 = 1 THEN L56
calc v20 = v23 - 1
&incr A1
&GOTO L54
AL56 *
vsave close w4:dail
idris basic -147000 -r /usr/ir/data/com1.ba
do print
do pause 1
do sclear
&edef A1 ="st001"
calc v31 = 1
vretrieve open /usr/ir/data/mail.da
vretrieve open /usr/ir/data/mail.da a14
vretrieve close /usr/ir/data/mail.da
+A14
calc v30 =&A14
do print
do pause 1
AL57 *
&IF v30 =v31 THEN L60
calo v31 = 31 + 1
&incr a1
&GOTO L60
AL60 *
do sclear
AL65 *
do sclear
retrieve x F0:&A2
retrieve y w4:&A1
267
calc v3 = (x(v1))
calc v4 = (y(v1))
calc v5 = v3/v4
mult y &v5
calc v6 = (x(v2))
calc v7 = (y(v2))
calc v8 = v6 - v7
vsave w4:date v8
do ssolear
diff x y z 1
save z Pl: &A2
calc v54 = (z(v2))
golear
do ssolear
* &A2
*
HAVENUMBER USED TO DETERMINE FACTOR = &v1
*
FACTOR USED = &v5
*
BASELINE ABSORBANCE OF PEAK = &v7
*
ABSORBANCE OF PEAK &v2 = &v6
*
ABSORBANCE OF PEAK &v2 (baseline corrected) = &v8
*
ABSORBANCE OF DIFF PEAK &v2 = &v54
*

do print
do pause
do ssolear
&mr A2
calc v20 =v20 -1
&IF A17 ="y" THEN L65
&IF v20 =0 THEN L70
&GOTO L53

L65 *
&IF A21 ="plot" THEN L66
&IF A21 ="print" THEN L67
AL66 *
do ssolear
calo v10 =&A4
calo v11 =0
calo v12 = (x(v10))
calo v40 =5
plot abs6 &v40
plot asc6 &v11 &v12
plot x &A4 &A5 a
plot y &A4 &A5 a over
do ssolear
AL67 *
do ssolear
golear
view z &A4 &A5 a aute


axis
do sclear
gprint
gclear
&IF v20 = 0 THEN L70
&AGOTO L53
&L70 =
vsave close w4:dat
&AGOTO L40
&L90 =
do sclear
gclear
*HAVE YOU ENTERED YOUR CONCENTRATION (X) VALUES
*TYPE y OR n AND ENTER
&ENTER All
&IF All = "y" THEN L96
&IF All = "n" THEN L95
&L95 =
*ENTER NUMBER OF SOLUTIONS (OR X VALUES)
&ENTER a8
calc v20 = a8
do sclear
idris rm /usr/ir/data/data.da
vsave open new:dat
do sclear
&L96 =
calo v12 = 1
*ENTER CONCENTRATION (X VALUES)
&ENTER A15
do sclear
vsave w4:dat a15
calo v20 = v20-1
&IF v20 = 0 THEN L97
&AGOTO L96
do sclear
&L97 =
vsave close w4:dat
&L98 =
idris basic -147000 -r /usr/ir/data/tab.ba do print
do pause 5
do sclear
idris basic -147000 -r /usr/ir/data/ploto.ba print
idris fullscreen
&AGOTO L999
do sclear
&L999 =
*END
do sclear
**NOISEOY**

* THIS PROGRAM WILL CALCULATE THE FOLLOWING NOISE VALUES IN VARIOUS REGIONS:
  * A. RMS
  * B. DIFFERENCE BETWEEN MINIMA AND MAXIMA
  * C. MEAN VALUE OF REGION
  * D. S/N (RMS)
  * E. S/N (PEAK-TO-PEAK)

* (NOTE USED LINEAR TREND REMOVAL FUNCTION)

* THIS PROGRAM WILL PRINT RESULT SO MAKE SURE THE EPSON PRINTER IS CONNECTED

do pause

do sclear
* ENTER NAME OF SPECTRAL FILE (SPECTRA SHOULD BE STORED ON A FLOPPY DISC IN PORT X AND SHOULD BE IN THE FORM XX001, WHERE X IS ANY LETTER, THIS WILL ALLOW THE PROGRAM TO RETRIEVE THE SPECTRA SEQUENTIALLY.

&enter a1

do sclear
* DO YOU WISH TO VIEW THE SPECTRA? y or n

&enter a2

&IF a2 = "y" then L20

&IF a2 = "n" THEN L30

L20 *

do sclear
* ENTER HIGHER WAVELENGTH OF REGION YOU WISH TO VIEW

&ENTER A3

do sclear
* ENTER LOWER WAVELENGTH OF REGION YOU WISH TO VIEW

&ENTER A4

retrieve x f0: &a1

view x &a3 &a4 a auto

do sclear
axis

*MOVE CURSOR TO HIGHER WAVELENGTH (N1) OF REGION 1 AND PRESS "H" TO ENTER

+WAVELENGTH

&cursor v6

do sclear

*MOVE CURSOR TO LOWER WAVELENGTH (N2) OF REGION 1 AND PRESS "H" TO ENTER

+WAVELENGTH

&cursor v7

do sclear

*MOVE CURSOR TO HIGHER WAVELENGTH (N3) OF REGION 2 AND PRESS "H" TO ENTER

+WAVELENGTH

&cursor v8
dc clear
*MOVE CURSOR TO LOWER WAVENUMBER (N4) OF REGION 4 AND PRESS "H" TO ENTER
&cursor v9
dc clear
*MOVE CURSOR TO HIGHER WAVENUMBER (N5) OF REGION 3 AND PRESS "H" TO ENTER
&cursor v10
dc clear
*MOVE CURSOR TO LOWER WAVENUMBER (N6) OF REGION 3 AND PRESS "H" TO ENTER
&cursor v11
dc clear
*MOVE CURSOR TO HIGHER WAVENUMBER (N7) OF REGION 4 AND PRESS "H" TO ENTER
&cursor v12
dc clear
*MOVE CURSOR TO LOWER WAVENUMBER (N8) OF REGION 4 AND PRESS "H" TO ENTER
&cursor v13
dc clear
&goto L40
&L40 *
dc clear
output clear v9
*ENTER HIGHER WAVENUMBER VALUE (N1) OF REGION 1
&enter A6
calo v6 = &A6
dc clear
*ENTER LOWER WAVENUMBER VALUE (N2) OF REGION 1
&enter A7
calo v7 = &A7
dc clear
*ENTER HIGHER WAVENUMBER VALUE (N3) OF REGION 2
&enter A8
calo v8 = &A8
dc clear
*ENTER LOWER WAVENUMBER VALUE (N4) OF REGION 2
&enter A9
calo v9 = &A9
dc clear
*ENTER HIGHER WAVENUMBER VALUE (N5) OF REGION 3
&enter A10
calo v10 = &A10
dc clear
*ENTER LOWER WAVENUMBER VALUE (N6) OF REGION 3
&enter A11
calo v11 = &A11
dc clear
*ENTER HIGHER WAVENUMBER VALUE (N7) OF REGION 4
&enter A12
calo v12 = &A12
dc clear
*ENTER LOWER WAVENUMBER VALUE (N8) OF REGION 4
&enter A13
calo v13 = &A13
dc clear
&L40 *
*DO YOU WISH TO START AGAIN? y OR n
*IF YOU WISH TO FINISH TYPE "end"
calc v32 = v38
calc v33 = 1.414*v30/v38
calc v34 = 1.414*v35/v37
calc v40 = (v16 + v21)/2
calc v41 = 1.414*sqrt(v16^2 + v21^2)
calc v42 = (v17 * v22)/2
calc v43 = 1.414*sqrt(v17^2 + v22^2)
calc v44 = (v20 • v21)/2
calc v45 = 1.414*sqrt(v20^2 + v21^2)
calc v46 = (v27 + v32)/2
calc v47 = 1.414*sqrt(v27^2 + v32^2)
do sclear

*&A1*
*
REGION 1 &v6 &v7 &v15 &v16 &v17 &v18 &v19
REGION 2 &v8 &v9 &v20 &v21 &v22 &v23 &v24
REGION 3 &v10 &v11 &v25 &v26 &v27 &v28 &v29
REGION 4 &v12 &v13 &v30 &v31 &v32 &v33 &v34
*
mean of regions &v40 &v41 &v42 &v43
1 + 2
mean of regions &v44 &v45 &v46 &v47
3 + 4
*
do print
do pause 10
do sclear &incr A1
calc v14 = v14-1
&IF A14 = "y" THEN L65
&IF v14 = 0 THEN L70
&GOTO L66
&L65 =
do sclear
view x &A3 &A4 a suite
do sclear
axis gprint
gprint
do pause 70
&IF v14 = 0 THEN L70
&GOTO L66
&L70 =
&GOTO L40
&L100 =
do sclear
&L899 =
*END*
do sclear

273
&1100 *
output clear v0
output clear a0
gclear
do sclear
*ENTER NAME OF SPECTRAL FILE (SPECTRUM SHOULD BE STORED ON
*FLOPPY DISK PORT X)
&ENTER a1
do sclear
*ENTER NAME OF SPECTRAL FILE WHICH WILL RECEIVE DATA
&ENTER a2
do sclear
*ENTER NUMBER OF SPECTRA TO BE ANALYSED
&ENTER a3
calo v15 = &a3
&1125 =
do sclear
retrieve x f0:&a1
copy x x 1000 880 1.0
save x f1:&a1
calo v3 = 1000
calo v4 = 940
calo v3 = 880
calo v2 = 880
copy x y &v3 &v2 1.0
copy x z &v5 &v4 1.0
taat y
taat z
calo v13 = ynpts
calo v12 = znupts
* y = &v13 z = &v12
&L150 *
idris r /usr/ir/data/cubica.da
idris r /usr/ir/data/cubicb.da
vsave open new w4:cubica
vsave open new w4:cubicb
&L200 *
calo v6 = abs(y(&v2))
** &v6 &v2
vsave w4:cubica v2
vsave w4:cubicb v6
&if v2 = v3 then 1250
calo v2 = v2 + 1
&GOTO L200
&L250 *
calo v2 = v4
&L251 *
calo v6 = abs(z(&v2))
vsave w4:cubica v2
vsave w4:cubicb v6
&if v2 = v5 then 1200
calo v2 = v2+1
&GOTO L251
&L200 *
vsave close w4:cubica
vsave close w4:cubicb

idris basic -147000 -r /usr/ir/data/cubic.ba
obey w4:cubicc
calo v15 =v15-1
&if v15 =0 then 1999
&incr a1
&incr a2
&goto 1125
&1999 *
*END
do sclear
$10$
$12$ open "o", #8, "/dev/tty3"
$13$ rem x y data placed in array
$20$ dim idat(200,2)
$25$ dim s(200), a(200,4)
$30$ np = 0
$40$ open "i", #6, "/usr/ir/data/cubica.de"
$50$ open "i", #5, "/usr/ir/data/cubicb.da"
$60$ i = 1
$60$ input #6, t, idat(i,1)
$70$ input #5, b, idat(i,2)
$90$ i = i+1
$100$ np = np+1
$110$ if np=77 goto 115
$115$ goto 80
$120$ rem print #6, np
$130$ rem this routine computes the matrix for finding the coefficients
$140$ rem of a cubic spline through a set of data. The system is solved
$150$ rem to obtain the second derivative values.
$160$ rem parameters are:
$170$ rem x(idat(i,1), y(idat(i,2)) - array of x and y values to be fitted
$180$ rem s = array of second derivative values
$190$ rem np = number of points
$200$ rem iend = type of end conditions to be used
$205$ rem a = augment matrix of coefficients and rhs for finding s
$210$ nm2 = np-2
$220$ nm1 = np-1
$230$ dx1 = (idat(2,1)-idat(1,1))
$240$ dy1 = (idat(2,2)-idat(1,2))/dx1*6.0
$260$ for i = 1 to nm2
$280$ dx2 = (idat(i+2,1)-idat(i+1,1))
$290$ dy2 = (idat(i+2,2)-idat(i+1,2))/dx2*6.0
$300$ a(i,1) = dx1
$310$ a(i,2) = 2.0*(dx1 + dx2)
$330$ a(i,3) = (dx2*dx2-dx1*dx1)/dx2
$340$ next i
$350$ dxl = dx2
$360$ dy1 = dy2
$370$ next i
$380$ rem adjust first and last rows appropriate to last end conditions
$390$ rem for iend: cubic ends, s(1), s(n) are extrapolated
$400$ dx1 = (idat(2,1)-idat(1,1))
$410$ dx2 = (idat(3,1)-idat(2,1))
$420$ a(1,2) = (dx1 + dx2)*(dx1 + 2.0*dx2)/dx2
$430$ a(1,3) = (dx2*dx2-dx1*dx1)/dx2
$440$ dxm2 = (idat(nm1,1)-idat(nm2,1))
$450$ dxm1 = (idat(np,1)-idat(nm1,1))
$460$ a(nm2,1) = (dxml*dxm2 - dxm1*dxm1)/dxm2
$470$ a(nm2,2) = (dxm1 + dxm2) * (dxm1 + 2.0*dxm2)/dxm2
$490$ rem now we solve the tridiagonal system. first reduce
$500$ for i=2 to nm2
$510$ a(i,2) = a(i,2) - a(i,1)/a(i-1,2)*a(i-1,3)
$520$ a(i,4) = a(i,4) - a(i,1)*a(i-1,2)*a(i-1,4)

275
%1100 *
override open /usr/ir/data/cubicc
override open /usr/ir/data/cubicd
calc v27 = 915.0
&1150 *
override x F1:AA1
override x y
calc v29 = 1
set wait off
&1200 *
override /usr/ir/data/cubicc a20
override /usr/ir/data/cubicd a30
do solear
*
&V26 &A20 &A30
change y de &a20 &a30
&if v25 = 600 then 1300
calc v28 = v25 + 1
&goto 1300
&A1300 *
taat x
taat y
calc v28 = aber(x(&v27))
calc v29 = aber(y(&v27))
calc v30 = v28-v29
do solear
*
&A1 &A2 &V28 &V29 &V30
do print
do pause 4
do solear
gclear
taat x
taat y
view x a auto
view y a auto
axis
do solear
great
save y fl:&a2
calo v21 = ymin
calo v22 = xmax
calo v40 = 5
plot abs v40
plot axscale &v21 &v22
plot x 1000 880 a
plot y 1000 800 a over
do solear
override close /usr/ir/data/cubicc
override close /usr/ir/data/cubicd
&A1999 *
462 REM print #8,a(i,1),a(i,2),a(i,3),a(i,4)
465 NEXT i
470 REM we back substitute
475 a(nm2,4) = a(nm2,4)/a(nm2,2)
480 FOR i=1 TO nm2
485 i1=nm1-i
490 REM print #8,a(i1,2),a(i1,3),a(i1,4)
495 a(i1,4) = (a(i1,4)-a(i1,3)*a(i1+1,4))/a(i1,2)
500 NEXT i
505 REM now put the values into the s vector
510 FOR i=1 TO nm2
515 s(i+1) = a(i,4)
520 NEXT i
525 REM end conditions. cubic ends
530 s(1) = ((dx1+dx2)*s(2)-dx1*s(3))/dx2
535 s(np) = ((dxm2 + dxn1)*s(nm1) - dxm1*s(nm2))/dxn2
540 REM fit data
545 FOR i=1 TO np
550 REM print #8,i,np,s(i)
555 NEXT i
600 i=1
610 k=800
620 t=999.9000
630 OPEN "c",#3,"/usr/ir/data/cubic.co"
640 OPEN "o",#4,"/usr/ir/data/cubic.do"
650 IF k>idat(i,1) THEN i=i+1
655 IF k>t THEN GOTO 770
660 xx=k-idat(i,1)
665 hn=(idat(i+1,1)-idat(i,1))/hx
670 a1 = (s(i+1)-s(i))/(hx*hx)
680 b = s(i)/2
690 z = i+1
700 c1 = (idat(z,2)-idat(i,2))/hx-(hx*(2.0*s(i)+s(z)))/6.0
710 di=idat(i,2)
720 REM print #6,i,s(i),b,c1,di
730 ycalc = (a1*((xx*xx)*xx)) + b*(xx*xx)+c1*xx+di
735 PRINT #3,"1","k"
740 PRINT #2,"1","ycalc"
745 REM print #6,idat(i,1),idat(i,2),k,ycalc
750 GOTO 640
760 system
Qualitative and quantitative determination of the cis and trans fatty-acid content of fats and oils using FTIR

Volume Two

by

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The figures and plots referred to in Volume One of this thesis are presented here. This format has been adopted purely for ease of presentation. All figures are presented together in Section One of this volume, whilst the plots are presented in Section Two. The numbering sequence of the figures and plots use the prefix corresponding to the section in which they are discussed.
SECTION ONE
\[
\begin{align*}
\text{CH}_2\text{-OH} & \quad \text{HOOC-R}_1 & \quad \text{CH}_2\text{-OOCR}_1 \\
\text{CH}_2\text{-OH} + \text{HOOC-R}_2 & \xrightarrow{\text{Esterification}} \quad \text{CH}_2\text{-OOCR}_2 + 3\text{H}_2\text{O} \\
\text{CH}_2\text{-OH} & \quad \text{HOOC-R}_3 & \quad \text{CH}_2\text{-OOCR}_3
\end{align*}
\]

Glycerol  Fatty acids  Triglyceride

**Figure 1.1.1**

(Where \( R_1, R_2 \& R_3 \) may be the same or different fatty acid groups)

\[
\begin{align*}
\text{C} & \quad \text{H} \\
\text{R}_1 & \quad \text{H} \\
\text{C} & \quad \text{C} \\
\text{R}_2 & \quad \text{R}_2 \\
\text{C} & \quad \text{H} \\
\text{R}_1 & \quad \text{H}
\end{align*}
\]

Cis isomer  Trans isomer

**Figure 1.1.2**

Geometric isomers of monoethanoic acids
Figure 1.1.4.1; Possible minimum energy conformation of saturated fatty acids in the solid phase

Figure 1.1.4.2; Possible minimum energy conformation of trans unsaturated fatty acids in the solid phase

Figure 1.1.4.3; Possible minimum energy conformation of cis unsaturated fatty acids in the solid phase
Figure 1.2.1

Michelson interferometer
Figure 1.2.2; Example of constructive interference

Figure 1.2.3; Example of destructive interference
Figure 1.2.4

Interferogram
SP001; 11:1Δ10 liquid phase spectrum (see Table 2.1.1)
SP002; 12:1Δ11 liquid phase spectrum (Table 2.1.1)
SP003; 13:1Δ12 liquid phase spectrum (Table 2.1.1)
SP004; 14:1Δ9c liquid phase spectrum (Table 2.1.1)
SP005:14:1Δ9t liquid phase spectrum (Table 2.1.1)
SP006; 16:1Δ9c liquid phase spectrum (Table 2.1.1)
SP007; 16:1Δ9t liquid phase spectrum (Table 2.1.1)
SP008; 18:1Δ6c liquid phase spectrum (Table 2.1.1)
SP009; 18:1Δ6t liquid phase spectrum (Table 2.1.1)
SP010; 18:1A9c liquid phase spectrum (Table 2.1.1)
SP011; 18:1Δ9t liquid phase spectrum (Table 2.1.1)
SP012; 18:1Δ11c liquid phase spectrum (Table 2.1.1)
SP013; 18:1Δ11t liquid phase spectrum (Table 2.1.1)
SP014; 18:1Δ9c12(OH) liquid phase spectrum (Table 2.1.1)
SP015; 18:1Δ9t12(OH) liquid phase spectrum (Table 2.1.1)
SP016; 18:2Δ9c12c liquid phase spectrum (Table 2.1.1)
SP017; 18:2Δ9t12t liquid phase spectrum (Table 2.1.1)
SP018; 18:3Δ6c9c12c liquid phase spectrum (Table 2.1.1)
SP020: 19:1Δ10c liquid phase spectrum (Table 2.1.1)
SP021; 20:1Δ8c liquid phase spectrum (Table 2.1.1)
SP022; 20:1Δ11c liquid phase spectrum (Table 2.1.1)
SP023; 20:1Δ11t liquid phase spectrum (Table 2.1.1)
SP024; 20:2Δ11c14c liquid phase spectrum (Table 2.1.1)
SP025; 20:2Δ11t14t liquid phase spectrum (Table 2.1.1)
SP026; 20:3Δ8c11c14c liquid phase spectrum (Table 2.1.1)
SP027; 20:3Δ11c14c17c liquid phase spectrum (Table 2.1.1)
liquid phase spectrum (Table 2.1.1)
SP029; 22:1Δ13t liquid phase spectrum (Table 2.1.1)
SP030; 22:4Δ7c10c13c16c liquid phase spectrum (Table 2.1.1)
SP031; 24:1Δ15c liquid phase spectrum (Table 2.1.1)
Figure 2.2.1.1; Spectrum of methyl stearate in the region 4000 to 1600 cm\(^{-1}\), which is representative of saturated FAME's. Bands indicated are assigned in Table 2.2.1.1.
Figure 2.2.1.2: Spectrum of methyl stearate in the region 1600 to 400 cm\(^{-1}\), which is representative of saturated FAME's. Bands indicated are assigned in Table 2.2.1.2.
ZONES A B C D

CH₃ - (CH₂)ₓ - (CH₂) - COOCH₃

FIGURE 2.2.1.3

Zones used to describe the assignment of various bands in saturated FAME's

Figure 2.3.1; Schematic diagram of the Perkin Elmer 1750 infrared Fourier transform spectrometer
Figure 2.3.8.1: Shows a Lorentzian band and corresponding interferogram.

Figure 2.3.8.2: Shows a wider Lorentzian band and corresponding interferogram, which decays more rapidly.

Figure 2.3.8.3: Illustrates deconvolution of band contours comprising of two overlapped 8cm\(^{-1}\) wide Lorentzian lines with relative intensities of 1 and 0.75. In figure A the lines are 6cm\(^{-1}\) apart. In figure B they are 2cm\(^{-1}\) apart.
Figure 2.3.10; Chromatogram showing unidentified FAME's produced during the isomerisation of methyl linoleate

\[
\begin{align*}
\alpha & \quad \beta \\
\text{CH}_3 - (\text{CH}_2)_n \cdot \text{CH}_2 - \text{CH} = \text{CH} - \text{CH}_2 - \text{CH} = \text{CH} - \text{CH}_2 - (\text{CH}_2)_n \cdot \text{COOCH}_3
\end{align*}
\]

FIGURE 2.4.1

Shows the assignments of the various categories of methylenes in poly-unsaturated FAME's
Figure 2.4.2.1.1; "Normal" spectrum of methyl oleate in the region 4000 to 2600 cm$^{-1}$

Figure 2.4.2.1.2; Deconvoluted spectrum of methyl oleate in the region 4000 to 2600 cm$^{-1}$
Figure 2.4.2.1.3; "Normal" spectrum of methyl stearate in the region 4000 to 2600 cm$^{-1}$

Figure 2.4.2.1.4; "Difference" spectrum obtained by interactively subtracting the spectrum of methyl stearate from that of methyl oleate.
Figure 2.4.2.2.1; Shows the spectrum of methyl linoleate (18:2Δ9c12c) in the region 4000 to 2600 cm⁻¹, which is representative of the di-unsaturated FAME's.

Figure 2.4.2.2.2; Deconvoluted spectrum of methyl linoleate in the region 4000 to 2600 cm⁻¹ (liquid phase).
Figure 2.4.2.2.3; Deconvoluted spectrum of methyl linoleate in the region 4000 to 2600cm⁻¹ (solution phase)

Figure 2.4.2.2.4; Deconvoluted solution spectrum of methyl vaccenate
(representative of cis mono-unsaturated FAME's)
Figure 2.4.2.2.5; "Normal" spectrum of 20:3Δ8c11c14c in the region 4000 to 2600cm\(^{-1}\)

Figure 2.4.2.2.6; Deconvoluted solution phase spectrum of 20:3Δ8c11c14c in the region 4000 to 2600cm\(^{-1}\)
Figure 2.4.2.2.7; Deconvoluted liquid phase spectrum of 20:3Δ8c11c14c in the region 4000 to 2600 cm\(^{-1}\)

Figure 2.4.2.2.8; Deconvoluted solution phase spectrum of 18:3Δ9c12c15c in the region 4000 to 2600 cm\(^{-1}\)
Figure 2.4.2.2.9; Deconvoluted spectrum of 22:4Δ7c10c13c16 in the region 4000 to 2600 cm⁻¹

Figure 2.4.2.2.10; Deconvolution solution phase spectrum of 18:4Δ6c9c12c15c in the region 4000 to 2600 cm⁻¹
Figure 2.4.2.2.11; Deconvolution solution phase spectrum of 22:6Δ4c7c10c13c16c19c

Figure 2.4.2.3.1; "Normal" spectrum of methyl elaidate in the region 4000 to 2600cm⁻¹
Figure 2.4.2.3.2: Deconvoluted spectrum of methyl elaidate in the region 4000 to 2600 cm\(^{-1}\).

\[ \text{CH}_3\text{C}==\text{O} \]

Plane of symmetry in plane of paper

Asymmetrical C-H stretching modes of the carbomethoxy group

Figure 2.4.2.3.3

Asymmetrical C-H stretching modes of the carbomethoxy group
Figure 2.4.2.3.4; "Normal" spectrum of 18:2Δ9t12t in the region 4000 to 2600 cm$^{-1}$

Figure 2.4.2.3.5; Deconvolution spectrum of 18:2Δ9t12t in the region 4000 to 2600 cm$^{-1}$
Figure 2.4.2.3.6; Spectrum of methyl-\textit{d}_3\textit{-stearate} (solution phase) in the region 3100 to 2970 cm\(^{-1}\), showing absence of the asymmetrical modes of the methoxy group.

Figure 2.4.2.3.7
Possible asymmetrical and symmetrical vibrational modes of the carbon hydrogen stretch across the double bond.
Figure 2.4.2.3.8; “Difference” spectrum obtained by interactively subtracting the spectrum of methyl stearate from that of methyl elaidate.

Figure 2.4.2.4; Shows the spectra of the 4th, 5th and 6th distillation fractions obtained from the isomerisation of methyl linoleate.
Figure 2.4.3.1: Differences in the profile of the cis C=C band occurring with change in the number of isolated cis double bonds.
Figure 2.4.3.2; Deconvoluted spectrum of 18:1Δ9c in the region of the C=C band, representing cis mono-unsaturated FAME's.

Figure 2.4.3.3; Deconvoluted spectrum of 18:2Δ9c12c in the region of the C=C bands, representing cis di-unsaturated FAME's.
Figure 2.4.3.4; Deconvoluted spectrum of 18:3Δ6c9c12c in the region of the C=C bands, representing one type of cis tri-unsaturated FAME's

Figure 2.4.3.5; Deconvoluted spectrum of 18:3Δ9c12c15c in the region of the C=C bands, representing other type of cis tri-unsaturated FAME's
Figure 2.4.3.6; Deconvoluted spectrum of 18:4Δ6c9c12c15c in the region of the C=C bands, representing one type of cis tri-unsaturated FAME's

Figure 2.4.3.7; Deconvoluted spectrum of 20:4Δ5c8c11c14c in the region of the C=C bands, representing other type of cis tri-unsaturated FAME's
Figure 2.4.3.8; Deconvoluted spectrum of 22:6Δ4c7c10c13c16c19c in the region of the C=C bands

Figure 2.4.3.9; Possible interactions between the C=C bonds in di- and tri-unsaturated cis FAME's
Figure 2.4.3.10: Cis and trans C=C stretching bands overlapped (carbonyl band subtracted)

Figure 2.4.3.11: Spectrum of the 7th distillation fraction from the alkaline isomerisation experiment
Figure 2.4.4.1; Deconvoluted spectrum of 18:1Δ9c in the region 1600 to 1000 cm\(^{-1}\)

Figure 2.4.4.2; Deconvoluted spectrum of 18:2Δ9c12c in the region 1600 to 1000 cm\(^{-1}\)
Figure 2.4.4.3; Deconvoluted spectrum of 18:3Δ9c12c15c in the region 1600 to 1000 cm\(^{-1}\)

Figure 2.4.4.4; Deconvoluted spectrum of 22:4Δ7c10c13c16c in the region 1600 to 1000 cm\(^{-1}\)
Figure 2.4.4.5; Deconvoluted spectrum of 18:1Δ9t in the region 1600 to 1000 cm\(^{-1}\)

Figure 2.4.4.6; Spectra of various mono-unsaturated FAME's, showing spectral similarities of bands in the region 1150 to 1050 cm\(^{-1}\) in both cis and trans FAME’s having identical numbers of methylenes in the chain attached to the carboxyl group.

Group of FAME’s having seven methylenes in the chain attached to the carboxyl group

Group of FAME’s having nine methylenes in the chain attached to the carboxyl group
Figure 2.4.5.1; Spectrum of methyl stearate in the region 950 to 760 cm⁻¹

Figure 2.4.5.2; Spectrum of CH₃-(CH₂)₁₆-COOCD₃ in the region 950 to 760 cm⁻¹
Figure 2.4.5.3; Deconvoluted spectra of various trans mono-unsaturated FAME's, showing spectral similarities of bands in the region 950 to 750 cm⁻¹ in trans FAME's having identical numbers of methylenes in the chain attached to the carboxyl group.
Figure 2.4.5.4; Deconvoluted spectra of 18:1Δ9c and 18:1Δ9t, showing spectral differences in the region 950 to 750 cm⁻¹ between cis and trans FAME's with equivalent numbers of methylenes in the chain attached to the carboxyl group.
Figure 2.4.5.5; Deconvoluted spectra of 18:2Δ9c12c and 18:3Δ6c9c12c in the region 950 to 750 cm⁻¹, illustrating the presence of a band at 913 cm⁻¹ in cis isolated polyunsaturated FAME's.
Figure 2.4.5.6; Spectra illustrating how the intensity of the band at 913 cm⁻¹ decreases with increase in the degree of conjugation in mixtures obtained from the distillation of alkaline isomerised methyl linoleate.

Figure 2.4.5.7; Spectra showing how the bands assigned to the \( \equiv \text{C-H} \) deformation of the conjugated cis/trans isomers of methyl linoleate increase in intensity with increase in conjugation.
Figure 2.4.5.8; "Normal" and deconvoluted spectra of methyl oleate in the region of the cis =C-H out-of-plane deformation.

Figure 2.4.5.9; "Normal" and deconvoluted spectra of methyl linoleate in the region of the cis =C-H out-of-plane deformation.
Figure 3.2.1.1; "Baseline" technique applied by Jackson et al. (1951) to calculate the trans band absorbance at 969 cm$^{-1}$

Figure 3.3.3.1; Spectra of chloroform and methyl elaidate superimposed, showing bands used to subtract solvent component from solution spectra
**Figure 3.3.3.2:** Spectra of tetrachloromethane and methyl elaidate superimposed, showing bands used to subtract solvent component from solution spectra.

**Figure 3.3.4.1:** Spectrum of trans FAME showing how the trans -C-H out-of-plane deformation peak height absorbance value is determined using the straight baseline correction technique.
Figure 3.3.5.1: Spectra of methyl oleate and methyl stearate (simulated baseline) in the region 1720 to 1600 cm\(^{-1}\), illustrating curved baseline simulated correction technique

- A3 = cis C=C peak maximum (1654 cm\(^{-1}\))
- A4 = absorbance of baseline spectrum at 1654 cm\(^{-1}\)
- A3 - A4 = cis C=C peak absorbance CBS baseline corrected
Figure 3.3.5.1; Spectrum of methyl oleate in the region 1720 to 1600cm⁻¹ (carbonyl band subtracted), illustrating peak area calculation using AREA.oy program.

Figure 3.4.1.1; Spectrum of chloroform taken with a 0.2mm pathlength cell.
regions in which resultant solute spectra are not observed

Figure 3.4.1.2; Spectrum of bromoform taken with a 0.5mm pathlength cell

regions in which resultant solute spectra are not observed

Figure 3.4.1.3; Spectrum of carbon tetrachloride taken with a 0.5mm pathlength cell
Figure 3.4.1.4; Typical noisy resultant solute spectrum of methyl elaidate after the subtraction of the solvent component (CCl₄). Obtained using a 0.2mm pathlength cell.

Figure 3.4.1.5; Effects of smoothing and flattening functions upon resultant solute spectrum of methyl elaidate. These functions were applied to the same solvent (CCl₄) and solution phase spectra used to obtain figure 3.4.1.4.
Figure 3.4.1.6: Resultant solute spectrum of the lowest concentration solution of methyl elaidate produced with 0.5mm cell (solvent - CCl₄). Spectrum shows interference fringes which contributed to the error in the trans band absorbance value.

Figure 3.4.1.7: Resultant solute spectrum of the lowest concentration solution of methyl elaidate retaken one week later using a 0.5mm cell (solvent - CCl₄) with frosted windows, which reduced the interference fringes observed in previous figure.
regions in which resultant solute spectra are not observed

Figure 3.4.1.8; Spectrum of carbon tetrachloride taken with a 1mm pathlength cell.
Figure 3.4.1.9; The solution and liquid phase spectra of methyl elaidate in the region 1500 to 900 cm\(^{-1}\) (1 mm cell).
Figure 3.4.3.1; Solution phase spectra of methyl palmitelaidate and methyl ricinelaidate; showing how the overlap of the hydroxy band affects the baseline used to determine the trans band absorbance.
Figure 3.4.3.2; Spectra of methyl stearate and methyl-d₃-stearate in the region 1050 to 900 cm⁻¹, showing how the methoxy vibrations are not present in the deuterated sample.
Figure 3.4.4.1; Solute spectrum of mixture a (methyl oleate - 38%; methyl elaidate - 62%). The baselines used to determine trans band absorbance are shown.

Figure 3.4.4.2; Solute spectrum of mixture e (methyl oleate - 97.5%; methyl elaidate - 2.5%). The baselines used to determine trans band absorbance are shown.
Figure 3.4.4.3; Liquid phase spectrum of methyl stearate.

Figure 3.4.4.4; Liquid phase spectrum of methyl oleate.
Figure 3.4.5.2.1; Capillary GC chromatogram of fat N88
Figure 3.4.5.3.1: Spectrum of 18:2Δ9c12c in the region 1150 to 650 cm⁻¹, illustrating how the spectral profile of bands in the trans band region vary.

Figure 3.4.5.3.2: Spectrum of 20:1Δ11c in the region 1150 to 650 cm⁻¹, illustrating how the spectral profile of bands in the trans band region vary.
Figure 3.4.5.3.3; Spectrum of 20:3\Delta11c14c17c in the region 1150 to 650 cm\(^{-1}\), illustrating how the spectral profile of bands in the trans band region vary.

Figure 3.4.5.3.4; Spectrum of 22:1\Delta13c in the region 1150 to 650 cm\(^{-1}\), illustrating how the spectral profile of bands in the trans band region vary.
concentrated solution of methyl elaidate

\[ N_1 = 976 \quad N_2 = 964 \]

dilute solution of methyl elaidate

\[ N_1 = 976 \quad N_2 = 964 \]

Figure 3.4.5.4.1; Solute spectra of methyl elaidate calibration solutions, illustrating how the baseline used to determine the peak area between the limits is not affected by the concentration of the solution.
Figure 3.4.5.4.2; Solute spectra of methyl elaidate/methyl oleate two component calibration mixtures, illustrating how the baseline used to determine the peak area between the limits is effected by the interfering bands present in methyl oleate.
Figure 3.4.6.1.1; Spectra of 18:0, 18:Δ9t and 18:2Δ9t12t, showing how the bands in the region of the cis =C-H stretch increase in intensity as the number of isolated trans double bonds increases.
Figure 3.4.6.2.1: Solvatochroic spectrum of a mixture of methyl oleate (2.5%) and methyl stearate (97.5%).

The spectrum shows two bands a and b, which are due to methyl stearate. These bands are also present in trans FAME's, and affect the straight baseline by increasing the absorbance at N1 and N3. The bands also obscure the cis band (ca. 3005 cm⁻¹) in mixtures of low cis content. The net effect is a low calculated cis content.
Figure 3.4.7.1.1: Resultant solute spectrum of methyl oleate in the region 1800 to 1600 cm$^{-1}$, illustrating the low intensity of the cis C=C stretching band.
Figure 3.4.7.2.1; Solute spectra of methyl oleate/methyl elaidate mixtures taken with 1mm and 2mm pathlength cells.
Figure 3.4.7.2.2: Resultant solute spectrum of a mixture of methyl oleate/methyl elaidate, illustrating how the trans C=C stretching band causes an error in the baseline used to determine the cis C=C band absorbance (spectrum produced with 2mm cell).
Figure 3.4.7.2.3; Shows the resultant solute spectrum of methyl stearate in the region 1700 to 1600 cm\(^{-1}\).
A3 - A2 = cis peak absorbance (baseline corrected); since A2 - A3 the cis peak absorbance is negative. Thus, resulting in a negative cis content.

Figure 3.4.7.2.4: Resultant solute spectrum of a mixture of methyl oleate and methyl stearate, illustrating the failure of the straight baseline correction technique when used to determine the cis C=C band absorbance.
Figure 3.4.7.4.1: Spectra showing how the curvature of the carbonyl shoulder of methyl stearate changes with concentration of the solution used. Spectra of methyl stearate were normalised to illustrate the effect.
Figure 3.4.7.4.2; Spectra showing how the curvature of the carbonyl shoulder of methyl oleate changes with concentration of the solution used. Spectra of methyl oleate were normalised to illustrate the effect.
Figure 3.4.7.4.3: Spectrum of methyl oleate in the region of the cis C=C band with the superimposed simulated baseline spectrum (methyl stearate) obtained using CBS technique.
Figure 3.4.7.4.4; C=C stretching bands of methyl elaidate and methyl oleate before and after subtraction of the carbonyl band component using the CBS technique.
Figure 3.4.7.5.1; Possible hydrogen bonding between hydroxyl group and cis C=C double bond.
Figure 3.4.7.6.1; Spectra of various mixtures of methyl oleate and methyl stearate with superimposed baseline spectra produced using the CBS technique. Solution spectra produced with 1mm pathlength cell.
Figure 3.4.7.6.2; Spectra of various mixtures of methyl oleate and methyl stearate with superimposed baseline spectra produced using the CBS technique. Solution spectra produced with 2mm pathlength cell.
Figure 3.4.7.9.1; FAME spectrum of fat B72E (liquid phase)
Figure 3.4.7.9.2: FAME spectrum of trial fat (liquid phase)
Figure 3.4.7.9.3; FAME solute spectrum of fat B72E taken with 2mm cell, plus simulated baseline spectrum produced by CBS technique superimposed.
Figure 3.4.7.9.4; FAME solute spectrum of trial fat taken with 2mm cell, plus simulated baseline spectrum produced by CBS technique superimposed.
Figure 3.4.7.9.5; Sunflower oil FAME liquid phase spectrum

Figure 3.4.7.9.6; FAME solute spectrum of sunflower oil taken with 2mm cell, plus simulated baseline spectrum produced by CBS technique superimposed.
**Figure 3.4.7.9.7;** Gamma oil FAME liquid phase spectrum

**Figure 3.4.7.9.8;** FAME solute spectrum of gamma oil taken with 2mm cell, plus simulated baseline spectrum produced by CBS technique superimposed.
Figure 3.4.7.9.9; Spectra of sunflower oil and gamma oil superimposed in the region of the cis C=C bands.

Figure 3.4.7.9.10; Spectrum of fat B72E FAME solution in the region of the cis C=C bands.
Figure 3.4.7.10.1; Examples of good and poor CBS baseline fit of solute FAME spectra of fat N88.
Figure 3.4.7.13.1; Example of peak area measurement of cis C=C band. The carbonyl band component was subtracted from the spectrum of methyl oleate before the AREA program was used to determine the area. Baseline parameters used were N1 = 1663; N2 = 1643.

Figure 3.4.7.13.2; Example of peak area measurement of cis C=C band. The carbonyl band component was subtracted from the spectrum of methyl oleate before the AREA program was used to determine the area. Baseline parameters used were N1 = 1669; N2 = 1640.
Figure 3.4.7.13.3; Example of peak height measurement of cis =C-H stretching band using the straight baseline correction technique. Baseline parameters used were $N1 = 3036\text{cm}^{-1}$, $N2 = \text{maximum}$, $N3 = 2986\text{cm}^{-1}$.
Resultant solute spectrum of FAME solution of FT001 (high in trans content) after subtraction of the carbonyl band

Figure 3.4.7.13.4; Example of the failure of the cis C=C stretching band peak area measurement using either baseline parameter set, caused by the high trans content of the sample.

Resultant solute spectrum of FAME solution of FT003 (high in poly-unsaturated FAME's) after subtraction of the carbonyl band

Figure 3.4.7.13.5; Example of the failure of the cis C=C stretching band peak area measurement using either baseline parameter set, caused by the high cis poly-unsaturated content of the sample.
Figure 3.4.3.1: Superimposed spectra of methyl linoleate and the simulated cubic spline baseline in the region 1000 to 800 cm\(^{-1}\).
Figure 4.3.1: Schematic drawing of the RIIC heated gas cell.

Figure 4.3.2: Schematic diagram of the Perkin Elmer GC-FTIR system.
Figure 4.3.2.2; Schematic of the connection of heated line to a gas chromatograph with a capillary column.

Figure 4.3.2.3; Schematic diagram of the gas flow in the GC-FTIR optical module.
Figure 4.4.1.1: Spectrum of methyl elaidate in the vapour phase

Figure 4.4.1.2: Spectrum of methyl elaidate in the vapour phase after subtraction of background spectrum
Figure 4.4.2.1; IR chromatogram of mixture (A)
(methyl oleate - 90.7%; methyl elaidate - 9.3%)

Figure 4.4.2.2; FID chromatogram of mixture (A)
(methyl oleate - 90.7%; methyl elaidate - 9.3%)

116
Figure 4.4.2.3: Best vapour phase spectrum obtained from IR chromatogram of mixture(A)
Figure 4.4.2.4; IR chromatogram of mixture(B)
(mixture of saturated methyl esters - C₆, C₁₀, C₁₂, C₁₄ and C₁₆)

Figure 4.4.2.5; FID chromatogram of mixture(B)
(mixture of saturated methyl esters - C₆, C₁₀, C₁₂, C₁₄ and C₁₆)
Figure 4.4.2.6: Vapour phase spectrum of C$_{14}$ obtained from IR chromatogram of mixture (B)
Figure 4.4.3.1; Vapour phase spectrum of DMF (14.8 ng) obtained on GC-FTIR system. A resolution of 8cm$^{-1}$ was used to produce the spectrum.

Figure 4.4.3.2; Vapour phase spectrum of DMF (10 ng) produced by Herres (1987). A resolution of 8cm$^{-1}$ was used to produce the spectrum.
Figure 4.4.5.1: Vapour phase spectrum of methyl elaidate

Figure 4.4.5.2: Vapour phase spectrum of methyl oleate
Figure 4.4.5.3; Vapour phase spectrum of methyl stearate

Figure 4.4.5.4; Vapour phase spectrum of methyl linoleate

122
Figure 4.4.5.5; Vapour phase spectrum of methyl linolenate

Figure 4.4.5.6; Vapour phase spectrum of methyl gamma linolenate
Figure 4.4.5.7: Liquid and vapour phase spectra of methyl elaidate superimposed
regions in which recognisable FAME spectra were obtained

Figure 4.4.6.1; FID and IR chromatograms of 0.4 μl injection of fat N88 (FAME solution)
Figure 4.4.6.2; FID and IR chromatograms of 1.0 μl injection of fat N88 (FAME solution)
Figure 4.4.6.3; Spectrum of main peak in IR chromatogram of fat N88 FAME solution (1 µl injection)
Figure 4.4.7.2.1: FID and IR chromatograms of a mixture of saturated FAME's obtained using the optimum conditions for the wide bore capillary column GC-FTIR system.

* region in which spectral data is obtained using FID trigger
Figure 4.4.7.2.2: Co-added and best individual spectra of a mixture of saturated FAME's obtained using the wide bore capillary column GC-FTIR system.
Figure 4.4.8.1: Spectrum showing how the absorbance measurement of various bands were determined.
SECTION TWO
Plot 3.4.3.1: Extinction coefficient of trans band ($k_1 = \text{moles/litre}$) versus number of carbons in fatty acid chain length of various trans mono-unsaturated FAME's.

Plot 3.4.3.2: Extinction coefficient of trans band ($k_2 = \text{grams/litre}$) versus number of carbons in fatty acid chain length of various trans mono-unsaturated FAME's.
Plot 4.4.8.1.1; Effect of spectral resolution on the cis =C-H/C=O band ratio of methyl oleate. Arrows represent the standard deviation (or error) of the band ratio calculated from the P-T-P noise.

Plot 4.4.8.1.2; Effect of spectral resolution on the cis =C-H/C=O band ratio of methyl oleate. Arrows represent the standard deviation (or error) of the band ratio calculated from the RMS noise.
Plot 4.4.8.1.3; Effect of spectral resolution on the C-H/C=O band ratio of methyl oleate. Arrows represent the standard deviation (or error) of the band ratio calculated from the P-T-P noise.

Plot 4.4.8.1.4; Effect of oven temperature on the cis-C-H/C=O band ratio of methyl oleate. Arrows represent the standard deviation (or error) of the band ratio calculated from the P-T-P noise. Values adjacent to points represent quantity injected onto column.
Plot 4.4.8.1.5; Effect of oven temperature on the C-H/C=O band ratio of methyl oleate. Arrows represent the standard deviation (or error) of the band ratio calculated from the P-T-P noise. Values adjacent to points represent quantity injected onto column.

Plot 4.4.8.3.1; The cis =C-H/C=O band ratios of various cis mono-unsaturated FAME's. Arrows represent the standard deviation (or error) of the band ratio calculated from the P-T-P noise.
Plot 4.4.8.3.2; The cis =C-H/C=O band ratios of various spectra of 18:2Δ9c12c taken on different occasions by injecting different quantities on the column. Quantities injected onto column represented by values adjacent to to points. diff refers to the resultant spectra produced after subtraction of a background spectrum.

Plot 4.4.8.3.3; The cis =C-H/C=O band ratios of various spectra of two different FAME's with four cis double bonds. Quantities injected onto column represented by values adjacent to points. diff refers to the resultant spectra produced after subtraction of a background spectrum.
Plot 4.4.8.3.4; Relationship between the cis =C-H/C=O band ratio and the number of isolated double bonds in the FAME.

Plot 4.4.8.3.5; Relationship between the C-H/C=O band ratio and the number of carbons in the fatty acid chain of FAME's.
Plot 4.4.8.3.6; Relationship between the C-H/C=C-O band ratio and the number of methylenes in the fatty acid chain of FAME's.

Plot 4.4.8.4.1; Relationship between the cis -C=C=O band ratio and the cis content of methyl oleate and methyl elaidate mixtures. Spectra were produced on wide bore GC-FTIR system using 1 μl injections.
Plot 4.4.8.4.2: Relationship between the \( \text{cis} = \text{C-H}/\text{C=O} \) band ratio and the cis content of methyl oleate and methyl elaidate mixtures. Spectra were produced on wide bore GC-FTIR system using 5 \( \mu \text{l} \) injections.

Plot 4.4.8.4.3: Relationship between the \( \text{trans} = \text{C-H}/\text{C=O} \) band ratio and the trans content of methyl oleate and methyl elaidate mixtures. Spectra were produced on wide bore GC-FTIR system using 1 \( \mu \text{l} \) injections.
Plot 4.4.8.4.4: Relationship between the trans \(\text{C-H/C=O}\) band ratio and the trans content of methyl oleate and methyl elaidate mixtures. Spectra were produced on wide bore GC-FTIR system using 5 \(\mu\)l injections.

Plot 4.4.8.4.5: Relationship between the cis \(\text{C-H/C=O}\) band ratio and the cis content of methyl oleate and methyl elaidate mixtures. Spectra were produced on wide bore GC-FTIR system using 1 \(\mu\)l injections. Diff indicates that a background spectrum was subtracted from the sample spectrum.
Plot 4.4.8.5.1: GC peak Homogeneity study of co-eluting geometrical isomers. Effect of cis =C-H,C=O band ratio across the GC peak of a mixture of methyl oleate and methyl elaidate. Spectra were produced on wide bore GC-FTIR system using 1 µl injections.

Plot 4.4.8.5.2: GC peak Homogeneity study of co-eluting geometrical isomers. Effect of trans =C-H,C=O band ratio across the GC peak of a mixture of methyl oleate and methyl elaidate. Spectra were produced on wide bore GC-FTIR system using 1 µl injections.
Plot 4.4.8.5.3; GC peak Homogeneity study of co-eluting geometrical isomers. Effect of C-H/C=O band ratio across the GC peak. Spectra were produced on wide bore GC-FTIR system using 1 μl injections.

Plot 4.4.8.5.4; GC peak Homogeneity study of co-eluting geometrical isomers. Effect of cis =C-H/C=O band ratio across the GC peak of a mixture of methyl oleate and methyl elaidate. Spectra were produced on wide bore GC-FTIR system using 5 μl injections.
Plot 4.4.8.5.5: GC peak Homogeneity study of co-eluting geometrical isomers. Effect of trans-C-H/C=O band ratio across the GC peak of a mixture of methyl oleate and methyl elaidate. Spectra were produced on wide bore GC-FTIR system using 5 µl injections.

Plot 4.4.8.5.6: GC peak Homogeneity study of co-eluting geometrical isomers. Effect of C-H/C=O band ratio across the GC peak. Spectra were produced on wide bore GC-FTIR system using 5 µl injections.
Plot 4.4.9.2.1; Cis = C-H/C=O band ratio vs. various quantities of "dil" and "conco" solutions of methyl oleate injected into the mega bore GC-FTIR system. Values adjacent to points represent quantities reaching light-pipe.

Plot 4.4.9.2.2; C-H/C=O band ratio vs. various quantities of "dil" and "conco" solutions of methyl oleate injected into the mega bore GC-FTIR system. Values adjacent to points represent quantities reaching light-pipe.
Plot 4.4.9.3.1: Relationship between the cis =C-H/C=O band ratio and the cis content of methyl oleate and methyl elaidate mixtures. Spectra were produced on mega bore GC-FTIR system using 1 μl injections.

Plot 4.4.9.3.2: Relationship between the cis =C-H/C=O band ratio and the cis content of methyl oleate and methyl elaidate mixtures. Spectra were produced on mega bore GC-FTIR system using 3 μl injections.
Plot 4.4.9.3.3: GC peak Homogeneity study of co-eluting geometrical isomers. Effect of cis =C-H/C=O band ratio across the GC peak of a mixture of methyl oleate and methyl elaidate. Spectra were produced on mega bore GC-FTIR system by injecting "dil" solution (1 µl).

Plot 4.4.9.3.4: GC peak Homogeneity study of co-eluting geometrical isomers. Effect of cis =C-H/C=O band ratio across the GC peak of a mixture of methyl oleate and methyl elaidate. Spectra were produced on mega bore GC-FTIR system by injecting "cono" solution (3 µl).