# The Determination of *Cis* and *Trans* Fatty Acid Isomers in Partially Hydrogenated Plant Oils

**By**

## Christiaan De Wet Marais

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at the

Department of Chemistry and Polymer Science
University of Stellenbosch


Study leader: Prof. A.M. Crouch
Department of Chemistry and Polymer Science
University of Stellenbosch

Co- study leader: Dr. C.M. Smuts
Nutritional Intervention Research Unit
The Medical Research Council
Tygerberg

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# DECLARATION

**I, Christiaan DeWet Marais, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.**




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# ABSTRACT

*Trans* isomers are formed during the partial hydrogenation process of *cis* unsaturated fatty acids. The major source of *trans* fatty acids in the normal person's diet is from margarines and shortenings made from these partially hydrogenated plant and marine oils. In addition to influencing lipid risk factors for cardiovascular disease, *trans* fatty acids have also been implicated in breast cancer, and in poor fetal development and reduced early infant growth. In reality, *trans* fatty acids have been consumed for centuries, since they occur naturally in beef, mutton, butter, milk and other dairy products. Though it has been shown that these naturally occurring *trans* fatty acids have different effects on the health of humans. With the implementation of the new labelling law in South Africa, the *trans* fatty acids content of food items must be displayed on the food label. Therefore, it becomes necessary to optimise the analytical methodology for the determination of *trans* fatty acids in foods.

Many publications have reported on the quantification of the total concentration of *trans* fatty acids in food samples, while less work has been done on the identification and quantification of the different *cis* and *trans* unsaturated fatty acid isomers found in foods made from partially hydrogenated oils. The objective of this study was to standardise and optimise an analytical technique to identify and quantify the different *cis* and *trans* mono-unsaturated fatty acid isomers in local margarines and bread spreads.

Seeing that fatty acids are the group of lipids most commonly analysed by GLC and the availability of highly polar capillary columns bonded with cyanoalkyl polysiloxan phases, it was decided to use GLC for the identification and quantification of the different *cis* and *trans* isomers in a selected group of margarines. It was further decided to evaluate two BPX-70 capillary columns packed with cyanoalkyl polysiloxan phases. The one a 30 m BPX-70 capillary column, normally used for routine fatty acid analyses, and the other a 120 m BPX-70 capillary column.

To extract the fatty acids from the samples, extraction solutions including chloroform, methanol and hexane were evaluated. For the transmethylation of the extracted fatty acids 0.5 M sodium methoxide in methanol and 5% concentrated sulphuric acid in methanol, were evaluated.

To optimise the GLC conditions, different column temperature programs and column gas flow rates were applied.

Of the three different extraction solutions evaluated in this study, chloroform/methanol (2:1) solution gave the best fatty acid recovery. It was also found that the 5% concentrated sulphuric acid/methanol transmethylation solution, gave a 7% better FAME recovery than 0.5 M sodium methoxide/methanol. When analysing a pooled margarine sample, it was found that with a 30 m BPX-70 capillary column the different *cis* and *trans* 18:1 isomers were forced to overlap due to the narrow elution gap, while a 120 m BPX-70 column provided the required mechanism for extending the retention times of the different isomers by retaining the different compounds longer. In this way, the retention times of the different isomers were pulled apart, and a greater separation space was available to identify more different isomers. It was found that column temperature had a major effect on the separation power of the 120 m BPX-70 capillary column. Isothermal operation at 181$^{o}$C produced the fewest overlapping peaks and 5 peaks could be separated before the main *cis*-9, 18:1 isomer and 7 peaks thereafter. Isothermal temperatures above and below 181$^{o}$C produce some additional overlapping problems.

The use of Ag-TLC separation before GLC analyses improves the identification of the different isomers, but it could not separate all the isomers, with the same geometrical structure that are eluting close together.

Using the optimised GLC conditions, eighteen different margarines were analysed. The results show that the normal occurring fatty acids, as well as most of the *cis* and *trans* fatty acids can be identified and quantified in one analytical run. The results further show that the *trans* fatty acid content of the selective group of local margarines are not as high as reported for some other countries, but that the saturated fatty acid content of these margarines is higher than the recommended levels.

Capillary electrophoresis was also utilised, but the separation and identification of the *cis* and *trans* fatty acid isomers in a standard sample were unsuccessful and much more analytical development is needed.

# OPSOMMING

*Trans* isomere word gevorm tydens die gedeeltelike hidrogenering van *cis* onversadigde vetsure. Die hoofbron van *trans* vetsure in die normale persoon se dieet word gevind in margarine en bakvet wat van gedeeltelik gehidrogeneerde plant en mariene olies vervaardig word. Buiten die effek wat *trans* vetsure op die lipied risiko faktore vir kardiovaskulêre siektes het, word dit verder verbind met borskanker, swak fetale ontwikkeling en vertraagde groei in die jong kind. In werklikheid word *trans* vetsure reeds vir eeue ingeneem aangesien hulle natuurlik in bees- en skaapvleis, botter, melk en ander suiwelprodukte voorkom. Daar is egter getoon dat hierdie natuurlike *trans* vetsure verskillende uitwerkings op die mens se gesondheid het. Die nuwe Wet op Etiketering in Suid-Afrika vereis dat die *trans* vetsuur inhoud van voedselitems op die voedseletiket vertoon moet word. Dit het daarom nodig geword om die analitiese metodologie vir die bepaling van *trans* vetsure in voedsels te optimaliseer.

Baie publikasies het al gerapporteer oor die bepaling van die totale konsentrasie van die *trans* vetsure in voedsel monsters, maar minder werk was gedoen op die identifisering en kwantifisering van die verskillende *cis* en *trans* onversadigde vetsuur isomere in voedsels wat vervaardig word van gedeeltelike hidrogeneerde plantolies. Die doel van hierdie studie was om ‘n analitiese tegniek te standardiseer en optimaliseer vir die identifisering en kwantifisering van die verskillende *cis* en *trans* mono-onversadigde vetsuur isomere in margarine en smere.

Omdat vetsure die groep lipiede is wat die mees algemeen deur GLC geanaliseer word, en omdat lang, hoogs polêre kapillêre kolomme, gebind met siano-alkiel polisiloksaan fases geredelik beskikbaar is, was daar besluit om GLC te gebruik vir die identifisering en kwantifisering van die verskillende *cis* en *trans* isomere in ’n uitgesoekte groep margarines. Daar was ook besluit om twee BPX-70 kapillêre kolomme, wat gepak is met siano-alkiel polisiloksaan fases, te evalueer. Die een, ’n 30 m BPX-70 kapillêre kolom, wat normaalweg vir roetine vetsuurbepalings gebruik word, en die ander, ’n 120 m BPX-70 kapillêre kolom.
Vir die vetsure ekstraheering van die monsters, ekstraksie oplossings wat insluit chloroform, metanol en hexaan was geevalueer. Vir die transmetelering van die geekstraheerde vetsure,

0.5 M natrium methoxide in metanol en 5% gekonsentreerde swaelsuur in methanol was geevalueer. Om die GLC kondisies te optimaliseer, verskillende kolom temperature en kolom gas vloei spoed, was getoets vir die analiseering van die margarine monsters.

Van die drie ekstraksie metodes wat geevalueer was het ‘n oplossing van chloroform/metanol (2:1) die beste vetsuur herwinning gegee. Daar is ook gevind dat 5% gekonsentreerde swaelsuur in metanol ‘n 7% beter herwinning van vetsuur metiel esters gegee het as 0.5 M natrium methoxide in methanol.

In ’n saamgestelde margarine monster is gevind dat met ’n 30 m kolom die verskillende *cis* en *trans* 18:1 isomere geforseer word om te oorvleuel as gevolg van die nou elueringsgaping, terwyl ’n 120 m kolom die kapasiteit het om die retensietye van die verskillende isomere te verleng deur die verskillende komponente langer terug te hou. Op hierdie manier is die retensietye van die verskillende isomere uitmekaar getrek, en is ‘n groter skeidingspasie beskikbaar vir die verskillende isomere. Hierdie skeidingskrag bring mee dat meer isomere geïdentifiseer kan word. Daar was gevind dat kolom temperatuur ‘n groot effek op die skeidingsvemoë van ‘n 120 m kapillêre kolom het. Met ‘n isotermiese temperatuur van 181$^{o}$C het die minste pieke geoorvleul en kon 5 pieke voor die hoof *cis*-9, 18:1 isomeer geskei word en 7 pieke daarna. Isotermiese temperature hoër en laer as 181$^{o}$C het additionele oorvleulingsprobleme veroorsaak.

Die gebruik van Ag dun-laagchromatografiese skeiding voor GLC analise, verbeter die identifisering van die verskillende isomere, maar kon nie al die isomere met dieselfde geometriese struktuur wat na aanmekaar elueer skei nie. Hierdie is as gevolg van die klein verskil in hulle onderskeie retensietye.

Deur gebruik te maak van die geoptimaliseerde GLC kondisies, is agtien verskillende margarines ontleed. Die resultate toon dat die vetsure wat gewoonlik voorkom, asook meeste van die *cis* en *trans* vetsure in een analitiese sessie geïdentifiseer en gekwantifiseer kan word. Die resultate toon verder dat die *trans* vetsuur inhoud van die geselekteerde groep plaaslike margarines nie so hoog is soos gerapporteer vir sommige ander lande nie, maar dat die versadige vetsuurinhoud van hierdie margarines hoër is as die aanbevole vlakke.

Kapillêre elektroforese is ook gebruik, maar die skeiding en identifisering van die *cis* en *trans* vetsuur-isomere in ’n standaardmonster was nie suksesvol nie, en verdere analitiese ontwikkeling word benodig.

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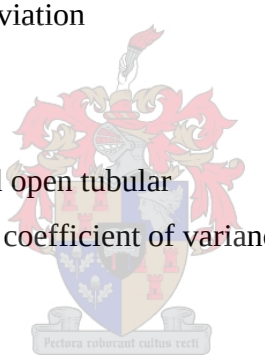
# LIST OF ABBREVIATIONS

| | |
|---|---|
| Ag-HPLC | High performance liquid chromatography with pack columns impregnated with silver nitrate |
| Ag-TLC | Silver nitrate impregnated thin layer chromatography |
| AOAC | Association of Official Analytical Chemists |
| AOCS | American Oil Chemists' Society |
| Avg | Average |
| Avg Rec | Average recovery |
| $BF_3$ | Boron trifluoride |
| BHT | Butylated hydroxytoluene |
| *c* | *Cis* |
| CHD | Coronary heart disease |
| CE | Capillary Electrophoresis |
| CEC | Capillary electrochemistry |
| CLA | Conjugated linoleic acids |
| C:M | Chloroform/methanol |
| $CS_2$ | Carbon disulfide |
| CZE | Capillary zone electrophoresis |
| DHA | Docosahexaenoic acid |
| EOF | Electroosmotic flow |
| EPA | Eicosapentaenioc acid |
| FAME | Fatty acid methyl ester |
| FID | Flame ionisation detector |
| FTIR | Fourier-transform infrared spectroscopy |
| GLC | Gas liquid chromatography |
| HDL | High-density lipoproteins |
| HPLC | High performance liquid chromatography |
| $H_2SO_4$ | Sulphuric acid |
| $H_2$ | Hydrogen |
| ID | Internal diameter |
| IR | Infrared spectroscopy |

| | |
|---|---|
| IUPAC | International Union of Pure and Applied Chemistry |
| LDL | Low-density lipoproteins |
| Lp (a) | Lipoprotein (a) |
| M | Mole |
| MEKC | Micellar electrokinetic chromatography |
| mM | Millimol |
| MUFA | Mono-unsaturated fatty acids |
| Na | Nano ampere |
| $NaOCH_3$ | Sodium methoxide |
| $N_2$ | Nitrogen |
| TFA | *Trans* fatty acids |
| TLC | Thin layer chromatography |
| SDS | Sodium dodecyl sulphate |
| STD | Standard deviation |
| *t* | *Trans* |
| UV | Ultra violet |
| WCOT | Wall-coated open tubular |
| % CV | Percentage coefficient of variance |



# CHAPTER 1

# INTRODUCTION AND AIMS OF THE STUDY

## 1.1 Introduction

The new labelling law of South Africa that will come into effect during 2006, states that the total concentration of the *trans* fatty acids in foods must be correctly displayed on food labels. (Draft Regulations Relating to Labelling and Advertising of Foodstuffs 2002, no R 1055). The major source of *trans* fatty acids in the human diet comes from margarines and shortenings made from partially hydrogenated plant and marine oils (Katan *et al*., 1995). It is well known that *trans* isomers are formed during the hydrogenation process of *cis* unsaturated fatty acids (Sommerfeld, 1983).

With the discovery that saturated fats have adverse effects on blood lipids, people turned to plant oils as a safe replacement for the saturated animal fats and butters used in cooking and table spreads. While few would question the health benefits of using some plant oils, it is the partially hydrogenated oils that have come under fire. The partial hydrogenation of plant oils improves the stability of the oil and makes it less likely to be oxidised. This process also converts the oil into a semi-solid fat. During the partial hydrogenation process, a variety of *cis* and *trans* fatty acid isomers are produced. The resulting fats and oils, in addition to containing *trans* fatty acids, have reduced amounts of the essential fatty acids, linoleic acid (18:2 n-6) and alfa-linolenic acid (18:3 n-3) (Emkin, 1995). These essential polyunsaturated fatty acids, with other unsaturated fatty acids are transformed, because they tend to oxidise easily, causing the oil to become rancid quite quickly. It has further been postulated that *trans* fatty acids in the circulating blood stream also inhibit the conversion of essential fatty acids, thereby increasing the requirements for essential fatty acid intake (Zevenbergen *et al*., 1988).

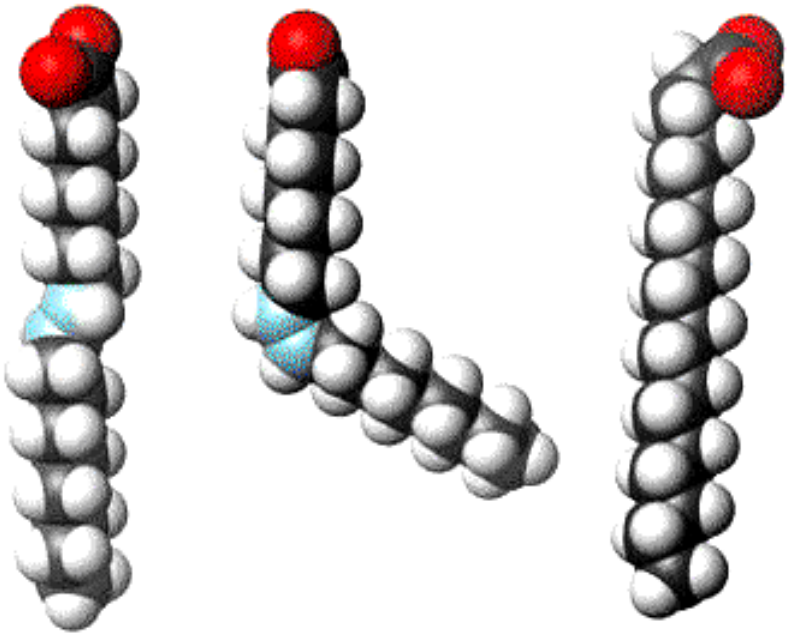
The current pressure to reduce the intake of saturated fat is probably promoting the consumption of partially hydrogenated plant oils and margarines that are likely to be high in *trans* fatty acids. Although the average level of *trans* fatty acids has declined with the advent

of softer margarines, per capita consumption of *trans* fatty acids has not changed greatly because of the increased use of commercially baked products and fast foods (Semma, 2002).

In reality, *trans* fatty acids have been consumed for centuries, since they occur naturally in beef, mutton, butter, milk and other dairy products (Parodi, 1976). They occur in animal fat largely because of the microbial hydrogenation in the animal rumen of the polyunsaturated fatty acids in the foods that animals feed on. *Trans* fatty acids have also been identified in very small amounts in some seeds and leafy vegetables (General Conference Nutrition Council, 2002).

The ingestion of *trans* fatty acids increases circulating low-density lipoproteins (LDL) to a degree similar to that of saturated fatty acids, but also reduces high-density lipoproteins (HDL). Saturated fatty acids do not affect the HDL, therefore *trans* fatty acids are considered more atherogenic than saturated fatty acids (Mensink *et al*., 1990). According to Dr. Stampfer, Professor of Nutrition at Harvard School of Public Health, *trans* fatty acids may be more dangerous to your health than saturated fatty acids. Studies in humans have found that *trans* fatty acids are about twice as bad as saturated fatty acids for your blood cholesterol and triacylglycerol levels (Strampfer, 2004). Mensink *et al*. (1990) published one of the first controlled intervention studies that specifically examined the effect of *trans* mono-unsaturated fatty acids (MUFA) from hydrogenated plant oils on the serum lipoprotein profile. From this study, it can be concluded that *trans* MUFA significantly raises serum total and LDL cholesterol concentrations and lowers HDL cholesterol, as compared with an iso-energetic amount of *cis* MUFA.

The main difference between *cis* and *trans* fatty acids isomers is in their geometrical structure. According to the structure resemblance between *trans* unsaturated fatty acids and saturated fatty acids and the differences in structure between *cis* and *trans* unsaturated fatty acids, one can assume that the *cis* positional isomers are not associated with coronary heart disease (CHD). These structural differences are demonstrated in Figure 1.



***Trans* unsaturated fatty acid** ***Cis* unsaturated fatty acid** **Saturated fatty acid**

**Figure 1. The geometrical structure of *trans* unsaturated, *cis* unsaturated and saturated fatty acids**

The geometrical structure of the *cis* isomers are bended, making it difficult to pack together tightly, while the structure of *trans* isomers are straight and very similar to that of saturated fatty acids making it possible to pack together tightly.

*Trans* fatty acids are well absorbed and it has been estimated that approximately 95% of *trans* MUFA are absorbed, which is similar to the rate of absorption of other fatty acids (Emkin, 1979). Other studies have shown that the position of the double bonds of *cis* and *trans* isomers have no effect on the absorption efficiency of these fatty acids (Emkin, 1997). After absorption, *trans* fatty acids follow the same metabolic routes as other fatty acids (Emkin, 1984).

Lipoprotein (a) (Lp (a)) concentrations in plasma have been associated with a higher risk for developing cardiovascular diseases (Lippi *et al*., 1999). After consumption of a meal high in *trans* fatty acids, blood Lp (a) concentrations have been reported to be increased in a number of publications (Lichtenstein *et al*., 1999; Sundram *et al*., 1997).

Another effect of *trans* fatty acid intake was published for the first time in 1961 by Anderson and his group. They noted that partially hydrogenated corn oil resulted in higher serum triglyceride levels than natural oils and butter (Anderson *et al*., 1961). A raising effect in

triglycerides was also seen in a number of recent studies that compared the effect of *trans* unsaturated fatty acids with *cis* unsaturated fatty acids in the blood lipid profile of humans (Lichtenstein *et al*., 1999; Sundram *et al*., 1997). No effect on serum triglyceride levels has been observed when substituting *cis* unsaturated fatty acids with saturated fatty acids (Mensink *et al*., 1992). Thus, *trans* fatty acids increase serum triglyceride levels when compared with other fatty acids.

In addition to influencing lipid risk factors for cardiovascular disease, *trans* fatty acids have also been implicated in breast cancer, and in poor faetal development and reduced early infant growth (Kohlmeier *et al*., 1997; Koletzko, 1992).

Presently, there is no specific method that permits analysts to distinguish between naturally occurring *trans* fatty acids and those produced industrially. This is because of the varying double-bond positions of *trans* fatty acid isomers in different hydrogenated oils (Wolff, 1995). Either infrared spectroscopy (IR) or gas liquid chromatography (GLC) is normally used to identify *trans* fatty acids in oils and fats. The IR method is not very reliable and lacks sensitivity for total *trans* fatty acid content below 5%. IR spectroscopy also does not distinguish individual *trans* fatty acids or detect positional isomers (Duchateua *et al*., 1996; Ulberth *et al*., 1996; Firestone *et al*., 1965). GLC can quantify the *trans* fatty acid contents as low as 0.01%, as well as identify some fatty acid isomers, assuming the analysts are well seasoned and using the latest available technology (Tang, 2002). The development of very long capillary columns coated with highly polar stationary phases has made it possible to separate some *cis* and *trans* fatty acid isomers (Christie, 1989). However, complete separation of all the *trans* and *cis* isomers is still very difficult with GLC analyses alone, as some isomers overlap. Identification can be improved by thin layer chromatography on silver nitrate impregnated silica plates (Ag-TLC) followed by GLC, but this method is very laborious (Christie, 1989; Molkentin *et al*., 1995). Good separations have been reported using long highly polar columns and the optimisation of the GLC oven temperature (Duchateua *et al*., 1996).

Complete separation and quantification of all the *cis* and *trans* isomers are necessary to provide accurate estimates of all the different fatty acid isomers in foods. This would allow the identification of the source of *trans* fatty acids in processed foods and mixed diets, as well

as the possible effects that different positional and geometrical isomers can have on diseases. Elaidic acid (*trans*-9, 18:1) is the major man-made *trans* fatty acid found in partially hydrogenated plant oils and processed foods, while vaccenic acid (*trans*-11, 18:1) occurs naturally in foods from animal sources. Because of their differences (specifically, the position of the double bond), they have very different physiological and biological effects on humans. (Belury, 2002). Mahfouz *et al*. (1984), found that feeding hydrogenated fat to animals decreases the conversion rate of linoleic acid to arachidonic acid because of the inhibitory effect that some *trans* fatty acid isomers have on delta 5 and delta 6 desaturase. In this study, the position of the *trans* double bond is shown to play a critical role in the degree of inhibition (Mahfouz *et al*., 1984). The identification of the position of the *trans* double bonds is also relevant because it has been suggested that *trans* fatty acids from dairy products, which have different positional *trans* double bonds, have different effects on the risk of CHD than those *trans* fatty acids from partially hydrogenated oils (Willet *et al*., 1995).

## 1.2 Aims of the study

The aims of the study are,

(1) To standardise and optimise an analytical technique to identify and quantify the different *cis* and *trans* mono-unsaturated fatty acid isomers in local margarines and bread spreads by GLC. Many publications have reported on the quantification of the total concentration of *trans* fatty acids in food samples, while less work has been done on the identification and quantification of the different *cis* and *trans* unsaturated fatty acid isomers found in foods made from partially hydrogenated oils.

(2) To evaluate the GLC results by using Capillary Electrophoresis (CE) for the analyses of the same samples on a comparative basis.

### 1.2.1 Specific objectives

To determine the best sample extraction method to use in the analyses of commercially available margarines and spreads.

- To determine the best transmethylation solution for use in preparing fatty acid methyl esters (FAME) of the extracted samples for GLC analyses.

To compare two different GLC column lengths for the identification and quantification of the different *cis* and *trans* fatty acid isomers.

- To optimise the GLC conditions to have a robust method.
- To identify and quantify as many *cis* and *trans* fatty acid isomers as possible.
- To use Ag-TLC for the separation of the *cis* and *trans* mono-unsaturated fatty acid fractions before GLC analyses, and to compare the results obtained with those obtained without Ag-TLC separation.
- To optimise CE conditions and develop a CE method that is faster.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Introduction

This study deals with the methodology to identify and quantify the different *cis* and *trans* fatty acid isomers in partially hydrogenated plant oils. The negative effects of some of the isomers on the health of humans are well known. From an analytical chemist's viewpoint, it is important to know the differences between commercially produced *cis* and *trans* fatty acids isomers and the natural occurring isomers, and how these can be identified and quantified. What analytical methodologies are available and how can these be improved?

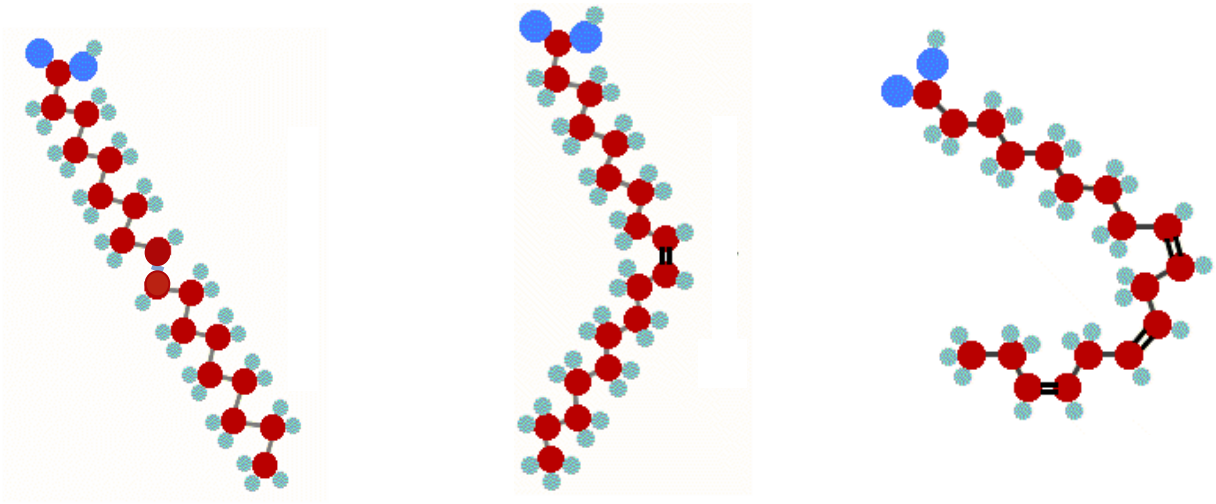
Work published thus far shows that the different positional and geometrical *trans* isomers have different effects on the health of humans (Mahfouz *et al*., 1984). It is a known fact that not all *trans* fatty acid isomers have a negative effect on the health of the population (Belury, 2002*).* For these reasons, an analytical technique using instrumentation that is normally available in a lipid analytical laboratory was researched to develop a technique to identify and quantify the different *cis* and *trans* isomers, as well as the other fatty acids that normally occur in partially hydrogenated oils.

## 2.2 Fatty acids

Fatty acids are a large and diverse group of naturally occurring organic compounds that are soluble in non-polar organic solvents (e.g., chloroform, ether, acetone and benzene) and generally insoluble in water. Fatty acids with up to six carbon atoms are considered short-chain fatty acids. They are more soluble in water than the longer chain fatty acids, and are therefore more easily digested and absorbed. Furthermore, they do not behave physiologically like the longer chain fatty acids, since they are more rapidly digested and absorbed in the intestinal tract. Biochemically, they are more closely related to carbohydrates than to fats. Fatty acids with eight to ten carbon atoms are said to have a medium chain. As for short-chain

fatty acids, studies have shown that intake of these medium-chain fatty acids may result in increased energy expenditure via fast digestion. They are further known to facilitate weight control when included in a diet as replacement for long-chain fatty acids (St-Onge *et al*., 2002). Fatty acids with 14 and more carbon atoms are considered as long-chain fatty acids.

The building blocks of most lipids are the fatty acids, which are essential for normal cell functioning and to stay healthy. They are composed of a chain of methylene groups with a carboxyl functional group at one end. The methyl chain is the fatty part, while the carboxyl group is the acid. Fatty acids can be saturated: all the carbon atoms have the maximum number of hydrogen atoms attached to them and have a straight-chain structure. Because of the straight structure of saturated fatty acid molecules, they can be packed tightly together, making them relatively dense and solid at room temperature. This cannot be altered by hydrogenation. They can also be unsaturated, with one or more double bond connecting some of the carbons. In unsaturated fatty acids, some of the carbon atoms miss some of their hydrogen atoms and thus form a double bond between those carbons missing their hydrogen atoms. With the formation of the double bond or bonds, a bend or kink is formed in the chain at these sites. The more double bonds an unsaturated fatty acid has, the more bended the molecule will be. Because of these bends or kinks, the molecules cannot stack together easily and stay fluid at room temperature. These are mostly oils. Figure 2 shows the geometrical structures of fatty acids with different degrees of saturation. Oils with a high percentage of saturated fatty acids are normally solid at room temperature. Other oils with a high percentage of mono-unsaturated fatty acids (with one double bond), such as olive oil, will solidify when cooled in a refrigerator. Polyunsaturated fatty acids, which have two or more double bonds and therefore more bends in their physical structure, stay fluid even when refrigerated.



**Saturated fatty acid** **Mono-unsaturated fatty acid** **Polyunsaturated fatty acid**

**Figure 2. Geometrical structures of fatty acids with different degrees of saturation**

When plants or animals make unsaturated fatty acids, they mostly make these kinked or bended forms: also referred to as *cis* unsaturated fatty acids. Most fatty acids are straight- or bended-chain compounds, and frequently have an even number of carbon atoms. Chain lengths can range from two to more than 80 carbon atoms, but commonly from 12 to 24. Branched-chain fatty acids are less common but are generally of microbial origin. These branched-chain fatty acids are usually not of any nutritional significance.

The common fatty acids in plant tissue are C16 and C18 with zero to three double bonds in the *cis* configuration. These fatty acids are also abundant in animal tissues, together with other fatty acids with a wider range of chain lengths and up to six *cis* double bonds separated by methylene groups. These methylene-interrupted double bonds are also referred to as non-conjugated double bonds. Figure 3 gives the chemical structure of a non-conjugated unsaturated fatty acid.

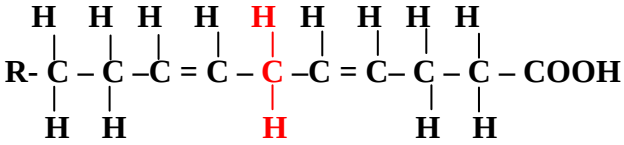



Figure 3. Non-conjugated polyunsaturated fatty acid structure

Polyunsaturated fatty acids can also be conjugated. Conjugated fatty acids do not have a methylene group between the two double-bonded carbons as can be seen in Figure 4.

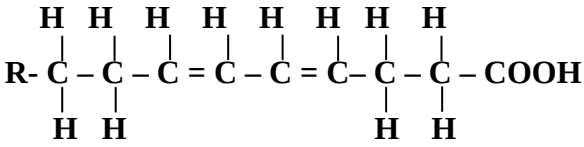


**Figure 4. Conjugated polyunsaturated fatty acid structure**

The most well known conjugated polyunsaturated fatty acids are probably conjugated linoleic acids (CLA). CLAs are a series of positional and geometrical isomers of linoleic acid (*cis*-9, *cis*-12, 18:2). Because of bacterial hydrogenation of linoleic acid in the animal's stomach, some of the double bonds flip over to the *trans* position and some even move to different positions on the carbon chain. However, the most distinctive reaction is the formation of conjugated double bonds. A number of *cis-cis*, *cis-trans*, *trans-cis*, and *trans-trans* isomers with the double bonds at various positions along the carbon chain have been identified. The *cis*-9, *trans*-11 isomer is the most abundant natural isomer present in ruminant fat (more than 90% of total CLA) (Christie, 2003). These *trans* conjugated polyunsaturated fatty acid isomers are not classified as *trans* fatty acids by the American Food and Drug Administration (Department of Health and Human Services, 2003).

Another group of natural occurring fatty acids are the omega-3 and omega-6 long-chain unsaturated fatty acids. The human body needs, but cannot synthesise these fatty acids and therefore they are called essential fatty acids. Essential fatty acids are very important, for example for our immune system. Alfa-linolenic acid (18:3, n-3) is the parent fatty acid of the omega-3 series. This is found in dark green vegetables and soybean oil, and is converted in the body to eicosapentaenioc acid (EPA) and docosahexaenoic acid (DHA). Marine algae and plankton also synthesise EPA and DHA, and therefore relatively high concentrations are found in the oil from fish that feed on algae. EPA and DHA also display several pharmacological properties, such as inhibition of inflammation, altered lipoprotein metabolism, inhibition of arteriosclerosis, decrease in blood pressure and inhibition of tumour growth (Sanders *et al*., 1997*).* In clinical trails, fish oil supplements containing EPA and DHA have also been shown to bring about some symptomatic relief in rheumatoid arthritis, colitis, psoriasis and Crohn's disease (Belluzzi *et al*., 1996).

Linoleic acid (18:2, n-6) is the parent fatty acid of the omega-6 series, and is the major fatty acid in sunflower and corn oils. This is converted to omega-6 fatty acids, mainly arachidonic acid (20:4, n-6) in the body. Arachidonic acid is also found in egg yolk and organ meats. Arachidonic acid can be converted into eicosanoids like prostaglandins, thromboxanes and leukotriens that are involved in the regulation of the actions of many cells. Most people eating a western diet that incorporates soft margarines and plant oils, such as sunflower, corn and peanut oil, get plenty of omega-6 fatty acids in their diets.

One of the main objectives of this study is to identify and quantify another group of fatty acids, namely, *trans* mono-unsaturated fatty acids.

## 2.3 *Trans* fatty acids

### 2.3.1 Natural occurring *trans* fatty acids

Most unsaturated fatty acids in nature have their double bonds in the *cis* configuration (Semma, 2002). Certain bacteria can covert these *cis* unsaturated fatty acids into unsaturated fatty acids with the double bonds in the *trans* configuration. In this configuration, some of the hydrogen atoms are on the opposite side of the double-bonded carbon atoms. This occurs in ruminants (cows, sheep and goats) where bacterial fermentation in the fore stomach causes the formation of *trans* unsaturated fatty acids. These isomers are found in the body fat of ruminants and in cows' milk and products, such as butter (Kepler *et al*., 1966; Mackie *et al*., 1991; Hay *et al*., 1970). *Trans* fatty acids which occur naturally in beef and dairy products have very different physiological and biological functions compared to man-made *trans* fatty acids that are found in processed foods (Belury, 2002). Data from the Nurses Health study reveal that while man-made *trans* fatty acids increase the risk of CHD, naturally occurring *trans* fatty acids of animal origin does not increase this risk (Willet *et al*., 1993).
Naturally occurring *trans* fatty acids have also been detected in the membrane lipids of various aerobic bacteria. Bacteria-degrading pollutants, such as Pseudomonas putida, are able to synthesise these compounds. They are synthesised by a direct isomerisation of the *cis* double bond without a shift in the position. This conversion changes the membrane fluidity in response to environmental stimuli (Keweloh *et al*., 1996).

Lamberto *et al*. have also identified two unusual *trans* fatty acids in seaweed that is grown in natural seawater. They identified *trans*-3, hexadecenoic acid (*trans*-3, 16:1) that has been known to occur as a component of plant photosynthetic lipids, and a novel *trans*-3, tetradecenoic acid (*trans*-3, 14:1) (Lamberto *et al*., 1994).

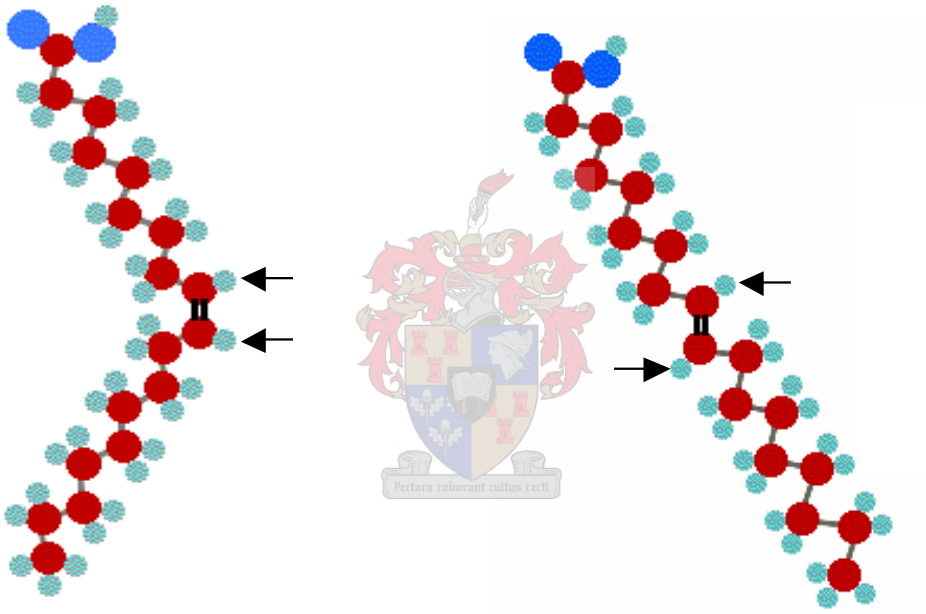
The most well-known group of natural occurring *trans* polyunsaturated fatty acids are probably conjugated linoleic acid (CLA). CLA is a collective term for a mixture of positional and geometrical isomers of linoleic acid, in which the two double bonds are conjugated. Dairy products are rich in CLA. Unlike the non-conjugated *trans* polyunsaturated fatty acids, CLA is recognised as possessing health benefits (Scimeca *et al*., 2000). This has been found to contain both antiatherogenic (Nicolosi *et al*., 1997) and anticarcinogenic properties (Ip *et al*., 1994). Micro-organisms in the rumen of animal converts *cis*-9, *cis*-12, octadecadienoic acid to mostly *cis*-9, *trans*-11, octadecadienoic acid and *trans*-10, *cis*-12, octadecadienoic acid, isomers. These two isomers are the most well-known *trans* conjugated polyunsaturated fatty acids (Parodi, 1997). These *trans* conjugated unsaturated fatty acids are not classified the same as the non-conjugated *trans* isomers that are formed during partial hydrogenation of plant and fish oils. Although these isomers include a *trans* configuration, they are not true *trans* fatty acids according to the definition of the American Food and Drug Administration, which defines *trans* fatty acids as ”unsaturated fatty acids that contain non-conjugated double bonds in a *trans* configuration”. (Department of Health and Human Services, 2003).

### 2.3.2 Commercially produced *trans* fatty acids

Except for the few cases described in the previous section, all other *trans* fatty acids are man-made. Wherever there is a double bond in a fatty acid chain, there is a possibility for the formation of both positional and/or geometrical isomers. With partial hydrogenation, a double bond may change from a *cis* position to a *trans* position (geometric isomerisation) or move to another position in the carbon chain (positional isomerisation) and both types of isomerisation may occur in the same molecule (Dutton, 1997*).*

Saturated fatty acids have a chain of carbon atoms joined by single bonds, allowing for rotation about the bonds. Naturally occurring unsaturated fatty acids contain double bonds of a particular configuration, referred to as *cis* unsaturated fatty acids. The double bond or bonds

restrict rotation. With partial hydrogenation some of these *cis* double bonds are converted to the *trans* isomer. Because the double bond restricts rotation, an unsaturated fatty acid can exist in two forms. The one is the *cis* form that has two parts of the carbon chain bended towards each other, with the hydrogen of the double bond on the same side of the chain (indicated by the two arrows in Figure 5). The other is the *trans* form that has two parts of the chain almost linear and with the two hydrogen atoms at the double bond on opposite sides of the chain (indicated by the two arrows in Figure 5).



***Cis* unsaturated fatty acid** ***Trans* unsaturated fatty acid**

**Figure 5. The geometrical structures of the *cis* and *trans* mono-unsaturated fatty acids**

The bended configuration of *cis* unsaturated fatty acids are the result of polarisation of the hydrogen atoms causing these to repel each other to form this bended chain. These bended configurations effectively prohibit the fatty acid molecules from packing tightly together. This means the bonds between the different *cis* molecules are weaker, resulting in the fat being either semi-solid with a low melting point or oil. Highly unsaturated vegetable oils are not suitable for many applications, such as margarines, shortenings and confectionary fats. The unsaturated oils are thus hardened by catalytic hydrogenation during which the naturally occurring *cis* unsaturated fatty acids are partly converted to the unnatural *trans* isomers. Depending on the type of unsaturated oil used and the temperature, pressure and duration of hydrogenation, different *trans* isomers can be formed. During hydrogenation, a few things can happen to the unsaturated oil. All the double bonds can be removed to form saturated fatty acids, or only some of the double bonds can be removed to change polyunsaturated fatty acids into monounsaturated fatty acids. Some of the double bonds may remain, but be moved in their positions on the carbon chain. Some of the *cis* double bonds can be changed into the *trans* position to produce several geometrical and positional isomers (Almendingen *et al*., 1995).

*Trans* fatty acids are well absorbed and incorporated into tissue lipids (Emken, 1995) and similarly transported to other fatty acids to be distributed within the cholesterol ester, triacylglycerol, and phospholipid fractions of the lipoproteins (Vidgren *et al*., 1998). The ingestion of *trans* unsaturated fatty acids increase low-density lipoproteins (LDL) to a similar degree of that of saturated fatty acids, but also reduces high-density lipoproteins (HDL). Therefore, *trans* fatty acids are considered to be more harmful than saturated fatty acids. (Ascherio *et al*., 1997). From the literature it is clear that the *trans* isomers of mono-unsaturated fatty acids are causing the negative attitude, rather than the *cis* positional isomers (Technical Committee of the Institute of Shortening and Edible Oils, 2006).

## 2.4 Hydrogenation

In the early 1900s, the only fat available for commercial use was lard, which was rendered easily and cheaply from pork fat. Lard has a good shelf live and excellent shortening properties, but is high in cholesterol and saturated fatty acids. With the growing health concerns about the dangers of eating too much saturated fat, there was pressure to find an

alternative, more unsaturated, source. During 1912, French chemist Paul Sabatier won the Nobel Prize for developing the hydrogenation process. This method allows oil refiners to modify unsaturated liquid oils to be suitable substitutes for lard (Paterson, 1996). A common misunderstanding is that partial hydrogenation changes all unsaturated fats to saturated fats. This is true for total hydrogenation, but with partial hydrogenation only some molecules of unsaturated fatty acids are converted to saturated fatty acids, while a large percentage of the natural occurring *cis* unsaturated fatty acids are converted to *trans* unsaturated fatty acid isomers.

Food manufacturers discovered that bubbling hydrogen through unsaturated oils, created partially hydrogenated fats that have a higher melting point and are less vulnerable to becoming rancid than the original oils and therefore have a longer shelf life. This process converts some of the *cis* or bended forms to a straightened or *trans* form. The chemical structure of the two forms is the same. It has the same number of carbon, oxygen and hydrogen atoms, and the double bond can be between the same two carbon atoms, but with a different geometrical configuration and it is a straight instead of kinked molecule because of the *trans* configuration. The body recognises the double bond and tries to use it for the same purposes that it uses the *cis* form, but the *trans* form stacks together just like saturated fatty acids, which sabotages the flexible and porous functionality of the cell membranes (Oslund-Lingvist *et al*., 1985).



Consider the differences between total hydrogenation and partial hydrogenation. If *cis*-9, *cis*-12, octadecadienoic (18:2), an unsaturated fatty acid with two double bonds in the *cis* positions and a melting point of -7$^{o}$C, is 100% hydrogenated, the two double bonds will be forced to break to form single bonds. An additional four hydrogen atoms will be added to the molecule. As a result of the total hydrogenation process, the bended molecule becomes a straight chain and the melting point of the oil will be changed to 70$^{o}$C and the structural configuration will resemble that of stearic acid (18:0). For all practical purposes this is a stearic acid. However, besides being costly it takes much energy to produce a saturated fatty acid that is naturally occurring and is also just too hard a fat to be made into margarine and shortening. Depending on the melting point of the fat that you need, you can partially hydrogenate the original oil to produce unsaturated oil with a specific melting point. For example, the partial hydrogenation of *cis*-9, *cis*-12, octadecadienoic fatty acids can produce

several different geometrical and positional isomers; only one double bond can break to give you *cis*-9, octadecenoic acid (18:1). It is still a bended molecule, but not as much as the original molecule, and its melting point is increased to 16$^{o}$C, or can change to the *trans*-9, octadecenoic acid isomer with a melting point of 44$^{o}$C and a straight geometrical structure. During the partial hydrogenation process, the double bond can even change to a different position on the carbon chain, for example to position 11 to give you *cis*-11, octadecenoic acid with a melting point of 12$^{o}$C and still be a bended structure, or to a *trans*-11, octadecenoic acid with a melting point of 39$^{o}$C, but again with a straight structure. All the different isomers of octadecenoic acid have the same molecular weight. While it is still an unsaturated fatty acid, it clearly is the number of double bonds as well as the positional and geometrical structure that determines the melting point of the final product. Vegetable oil is too soft to make margarine and shortening, and saturated fat is too hard. An in-between product is needed which is why the industry only partially hydrogenates the vegetable oils.

During the partial hydrogenation process, which is easily controlled, hydrogen atoms are added in no particular order. When the hydrogenation process is stopped, unsaturated fatty acids are in varying stages of hydrogenation. Some molecules are totally hydrogenated (saturated) while in others, some of the double bonds have changed from the natural *cis* configuration to the unnatural *trans* configuration. Some of the double bonds have even shifted to unnatural positions on the carbon chain. During the partial hydrogenation process, the bent *cis* isomer changes to the *trans* isomer forming a molecule that has a straight configuration, similar to saturated fatty acids. The straight configuration of *trans* unsaturated fatty acids enable the molecules to pack easily together resulting in a higher melting point with a longer shelf life and flavour stability. These more stable fats are used in margarines and shortenings.

## 2.5 Naming of fatty acids (Nomenclature)

Fatty acids are normally classified into two groups, either saturated or unsaturated. Unsaturated fatty acids can further be classified into monounsaturated, with one double bond or polyunsaturated with two or more double bonds. The unsaturated fatty acids derive their systematic names from the parent unsaturated hydrocarbon. The unsaturated fatty acid, octadecenoic acid (18:1) is derived from the hydrocarbon octadecene. The number of double

bonds in a polyunsaturated fatty acid chain is designated by the terms di-, tri-, tetra-, etc., inserted into the name as in octadeca***di***enoic acid (18:2) a polyunsaturated fatty acid with two double bonds and octadeca***tri***enoic acid (18:3), a polyunsaturated fatty acid with three double bonds. (Perkin, 1991). The numeric designations used for fatty acids come from the number of carbon atoms followed by the number of double bonds. To precisely describe the structure of a fatty acid molecule, the length of the carbon chain (number of carbons), the number of double bonds and also the exact positions of these double bonds must be known. This will define the biological reactivity of the fatty acid molecule.

According to official International Union of Pure and Applied Chemistry (IUPAC) nomenclature, the carbons in a fatty acid chain are numbered consecutively with the carbon of the carboxyl group being considered number one. This is also the form of nomenclature preferred by the International Commission on Biochemical Nomenclature. By convention, the lower number of two carbons that have a double bond identifies the first double bond in a chain. For example, in *cis*-9, octadecenoic acid (18:1) the double bond is between the $9^{th}$ and the $10^{th}$ carbon atom and it is a *cis* isomer. Another form of nomenclature designates octadecenoic acid as 18:1(n-9), which indicates that the double bond is 9 carbons away from the methyl group. Although this contradicts the convention that the position of the double bond should be counted from the carboxyl end of the carbon chain, it is of great convenience to lipid biochemists, because the number of the last double bond remains the same when carbon atoms are added or removed from the carboxyl end during metabolism (Nutritiondata, 2006).

The polyunsaturated fatty acid, cis-9, cis-12, octadecadienoic acid (linoleic acid), explains the nomenclature better. Counting the carbon atoms from the carboxyl group, the first double bond is between the $9^{th}$ and the $10^{th}$ carbon and the second double bond is between the $12^{th}$ and the $13^{th}$ carbon, and the hydrogen atoms on the carbon atoms, at both double bonds, are in the *cis* positions. Counting the carbon atoms from the methyl group the first double bond is between the $6^{th}$ and the $7^{th}$ carbon. This is why linoleic acid is also known as 18:2 (n-6), an omega-6 polyunsaturated fatty acid (Figure 6).

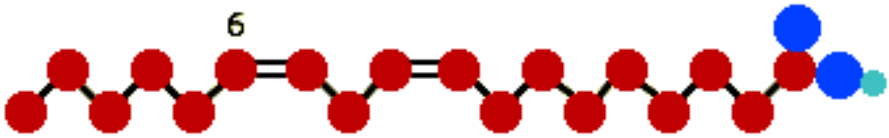



**Figure 6. Geometrical structure of *cis*-9, *cis*-12, octadecadienoic acid, also known as 18:2 (n-6)**

The aim of this study is to identify the different *cis* and *trans* fatty acids isomers. Therefore, the nomenclature as preferred by the IUPAC is the most appropriate. With this system, all the different positional and geometrical fatty acid isomers can be identified by their names.

The following list (Table 1) gives the scientific names, shorthand designation and the trivial name of some of the fatty acids used in this thesis.

These are just a few of the most common fatty acids. With partial hydrogenation, the natural monounsaturated and polyunsaturated fatty acids can form a number of different positional and geometrical fatty acid isomers each with their own name.

**Table 1. The scientific names, shorthand designation and trivial names of some of the fatty acids**

| Saturated fatty acids | | |
|---|---|---|
| **Scientific name** | **Shorthand designation** | **Trivial name** |
| Dodecanoic acid | 12:0 | Lauric acid |
| Tetradecanoic acid | 14:0 | Myristic acid |
| Hexadecanoic acid | 16:0 | Palmitic acid |
| Heptadecanoic acid | 17:0 | |
| Octadecanoic acid | 18:0 | Stearic acid |

| Monounsaturated fatty acids | | |
|---|---|---|
| **Scientific name** | **Shorthand designation** | **Trivial name** |
| *Cis*-9, Tetradecenoic acid | 9-14:1 | Myristoleic acid |
| *Cis*-9, Hexadecenoic acid | 9-16:1 | Palmitoleic acid |
| *Trans*-9, Hexadecenoic acid | 9-16:1 | Palmitelaidic acid |
| *Cis*-6, Octadecenoic acid | 6-18:1 | Petroselinic acid |
| *Cis*-9, Octadecenoic acid | 9-18:1 | Oleic acid |
| *Cis*-11, Octadecenoic acid | 1-18:1 | Vaccenic acid |
| *Trans*-6, Octadecenoic acid | 6-18:1 | Petroselaidic acid |
| *Trans*-9, Octadecenoic acid | 9-18:1 | Elaidic acid |
| *Trans*-11, Octadecenoic acid | 11-18:1 | Trans-vaccenic acid |

| ***Polyunsaturated fatty acids*** | | |
|---|---|---|
| **Scientific name** | **Shorthand designation** | **Trivial name** |
| *Cis*-9,*Cis*-12, Octadecadienoic acid | 9c,12c-18:2 | Linoleic acid |
| *Cis*-9,*Trans*-11, Octadecadienoic acid | 9c,11t-18:2 | Conjugated linoleic acid |

## 2.6 Analytical procedures for the determination of *cis* and *trans* fatty acids

### 2.6.1 Introduction

Currently there are two official methods for the quantification of *trans* fatty acids as accepted by the American Oil Chemists' Society (AOCS) and the Association of Official Analytical Chemists (AOAC), namely GLC and IR Spectroscopy. Several other analytical methods are reported for *trans* fatty acid determination and quantification in food. These analytical procedures mostly stem from separative techniques generally used for lipid analyses namely, Ag-TLC, high performance liquid chromatography (HPLC), high performance liquid chromatography with packed columns impregnated with silver nitrate (Ag-HPLC) as well as the two accepted methods. Each of these methods has advantages and drawbacks. Improvements in the accuracy and effectiveness of the results can be obtained by combining some of these methods.

### 2.6.2 Infrared spectroscopy

Infrared spectroscopy is the method that was used over the last few decades to determine the total *trans* fatty acid composition of food samples. *Trans* ethylenic bonds show a specific absorption in the infrared spectrum at 967 $cm^{-1}$. This method is fast and easy for routine analyses, but the IR method is not very reliable and lacks sensitivity for total *trans* fatty acid content below 5%. IR spectroscopy also does not distinguish individual *trans* fatty acid isomers or detect positional isomers (Duchateua *et al*., 1996). Furthermore, results obtained using IR spectroscopy is higher, sometimes as much as twice those obtained by GLC (Ulbrecht *et al*., 1994). Several reasons could explain these discrepancies. Most triacylglycerols are absorbed in the infrared spectrum at a similar wavelength as the *trans* isomers, which lead to an apparent increase in the *trans* fatty acid level measurements (Deman *et al*., 1983). IR spectroscopy also measures conjugated *trans* fatty acid isomers, which are not considered real *trans* fatty acids (Ulbrecht *et al*., 1994). Limitations in the use of IR spectroscopy to determine the *trans* fatty acids content of food samples were the lack of accuracy, especially at low levels of *trans* fatty acid isomer content, and the inability to distinguish between the different positional and geometrical isomers (Ulbrecht *et al*., 1996; Firestone *et al*., 1965). Emergence of Fourier-transform infrared spectroscopy (FTIR) and the

use of computer-assisted spectral subtraction procedures allowed for the improved detection efficiency of this method. Unfortunately, this method still yielded somewhat higher levels than the values recorded when using GLC. Furthermore, some large variations were noticed in the measurement of oils that contained low levels of *trans* fatty acids, as usually is the case with partially hydrogenated oils (Ulbrecht *et al*., 1994).

### 2.6.3 Silver impregnated thin layer chromatography

Geometric isomer separation using Ag-TLC is based on the property of *trans* isomers, which form unstable compounds in reaction to silver salts. These compounds are different from those formed with *cis* isomers (Ledoux *et al*., 2000). In most cases, the thin layer plates were dipped in a 5-20% silver nitrate solution, then dried and activated. The fatty acid methyl ester samples were then spotted and developed in saturated tanks in hexane-diethyl ether or petroleum ether- diethyl ether. This led to the separation of the *cis* and *trans* monounsaturated fatty acid fractions. The *cis* and *trans* monounsaturated fatty acid methyl ester spots were then scraped off the silica gel plates and analysed by GLC (Precht *et al*., 1997). GLC analyses after Ag-TLC, led to much better results than the use of GLC alone. Molkentin *et al*. (1995) succeeded in separating 10 peaks for *trans* 18:1 fatty acids and 9 peaks for *cis* 18:1 isomers using a 100 m CP Sil-88 capillary column after pre-separation by Ag-TLC. Ledoux and his group (2000) obtained 18 different peaks using similar operating conditions. This method has the drawback of being very time-consuming and laborious, with no possibility of automation. On the other hand, it is a cheap and easy method to use.

### 2.6.4 High performance liquid chromatography

The use of HPLC for the identification and quantification of different *cis* and *trans* fatty acid isomers is one of the newer methods. Juanèda (2002) published a paper on the use of a HPLC fitted with two reverse-phase columns for the separation of the *cis* and *trans* isomers, but GLC still had to be used to analyse the collected fractions. In this study, an expensive HPLC was used only to separate the *cis* and *trans* isomers, while a GLC was still needed for the identification and quantification of the different isomers. The appearance of commercial silver-ion columns for HPLC has caused a revival of this technique. A number of papers have been published on the use of silver-ion high-performance liquid chromatography to identify

isomeric *cis* and *trans* fatty acids over the past few years (Adlof, 1994, Ratnayake, 2004). Both the capital and running costs of this technique are much higher than that of GLC. The complex nature of the separation process causes the identification of compounds emerging from HPLC to be complicated (Christie, 1989).

### 2.6.5 Gas liquid chromatography

Fatty acids are the group of lipids most commonly analysed by GLC. It is undoubtedly the technique that would be mostly chosen for this purpose (Stoffel *et al*., 1959). The major advances in this method, regarding the identification and quantification of the different *cis* and *trans* fatty acids isomers, are the commercial availability of very long capillary columns packed with highly polar stationary phases. Column efficiency is proportional to the square root of column length, and resolution is influenced by the selectivity of the stationary phase. Increasing column length will therefore lead to higher resolution, and modification of the stationary phase will effect separation (Wolff *et al*., 1995).

Recently available highly polar columns bonded with cyanoalkyl polysiloxan phases, such as SP-2560 (Thompson, 1997) and BPX-70 (Berdeaux *et al*., 1998), demonstrated significant improvements in the separation and quantification of the different *cis* and *trans* isomers. By using cyanoalkyl polysiloxan as a stationary phase, *trans* 18:1 isomers are eluting in the double-bond position progression along the carbon chain from the carboxylic acid end of the fatty acid chain (*trans*-4, *trans*-5, *trans*-6, *trans*-7…). Most of the *trans* isomers also have shorter retention times than those of oleic acid (*cis*-9, 18:1) (Aro *et al*., 1998). Quantitation of the main *trans* isomer in milk fat, vaccenic acid (*trans*-11, 18:1) (Molkentin *et al*., 1995), together with *trans*-9, 18:1 and *trans*-10, 18:1, which represent the major *trans* isomers in hydrogenated plant oils (Parodi, 1976), can easily be done with these columns (Aro *et al*., 1998). From these chromatograms the source of the *trans* isomers in processed foods, can be identified. The superb resolutions attainable with the new very long highly polar, wall-coated open tubular (WCOT) capillary columns, make it more challenging to use GLC fitted with these columns for the identification and quantification of the different *cis* and *trans* fatty acid isomers in partially hydrogenated oil samples.

### 2.6.6 Capillary electrophoresis

Capillary electrophoresis is a highly efficient and flexible analytical separation technique that has become a serious competitor for GLC, but a number of problems remain to be solved. A very small sample volume is required for CE analyses. This can negatively impact precision and sensitivity. More importantly though, is the degree to which the small volume is representative of the overall sample, since it remains very problematic especially when working with oils with a low percentage of *trans* fatty acids (Castaneda *et al*., 2005). One way of minimising these problems and their strong effect on the quality of the results is to consider sample preparation as a key part of CE processes. (Valcarcel *et al*., 1998).

Fatty acids are normally analysed by GLC, but there is still a need to speed up the analytical time. An attractive alternative separation technique may possibly be CE, and in particular, micellar electrokinetic chromatography (MEKC) (Erim *et al*., 1995). An advantage of MEKC is the fact that compounds that are insoluble in aqueous solutions, like fatty acids, can be solubilised. The absence of a chromophoric or fluorophoric group in fatty acids excludes direct UV detection and therefore indirect detection has to be used (Erim *et al*., 1995). So far most of the articles on fatty acids with CE dealt with the analyses of saturated and unsaturated short, medium- and long-chain fatty acids. There were a few publications on *trans* fatty acids, but they dealt mostly with the identification of *cis* and *trans* isomeric groups and not so much on the identification of the different isomers. An article by de Oliveira *et al*. (2003) described a method to analyse *trans* fatty acids in hydrogenated oils by CE. They used indirect UV detection with sodium dodecyl benzenesulfonate as a chromophore and a neutral surfactant, polyethylene 23 lauryl ether. Elaidic acid (*trans*-9, 18:1) and oleic acid (*cis*-9, 18:1), as well as other saturated and unsaturated fatty acids were separated in hydrogenated Brazil nut oil (de Oliveira *et al*, 2003).

## 2.7 Lipid extractions

Very few papers deal with lipid extraction in depth, yet the correct extraction procedure is the first critical step in the identification and quantification of fatty acids. Quantitative isolation of all the lipids in the sample in their native state and which are free of contaminants must be accomplished before being analysed. Care must be taken to minimise the risk of hydrolyses

and oxidation of the fatty acids. To extract the fatty acids, it is necessary to find solvents that will not only dissolve the lipids readily, but will also overcome the interaction between the lipids and the sample matrix. Most lipid analysts use a mixture of chloroform and methanol to extract the lipids from animal and plant material (Christie, 1993). Over the years some interest has been shown in *iso*-propanol/hexane (2:3), because its toxicity is relatively low, but much more testing needs to be done on its extraction ability (Radin, 1981). Benzene was also frequently mentioned as a solvent with very good extraction properties. However, today this is known to be extremely toxic and other solvents are preferred, even though most solvents exhibit some degree of toxicity if inhaled.

Margarines consist mainly of triacylglycerol molecules with very little non-lipid contaminants, making the extraction procedure quite simple. Any lipid lacking polar groups, for example triacylglycerols, are soluble in moderately polar solvents such as chloroform, and very soluble in hydrocarbons such as hexane (Christie, 1993). Most of the literature describing the extraction of lipids from fat and oils mention the use of a mixture of chloroform and methanol that is based on the method first published by Folch *et al*. (1957). Bligh *et al*. (1959) published a simple adaptation of the original Folch method merely as an economical means of extracting lipids from fish. Others tried the Bligh method and found it lacking in the recovery of non-polar lipids (Cabrini *et al*., 1992). Richardson *et al*. (1997) also described a modification of Folch's extraction method that gave excellent results. These scientists used large volumes of chloroform and methanol, with a final ratio of 1:1(v:v), to extract the fatty acids and then used a rotary evaporator to remove the solvent (Richardson *et al*, 1997). Lepage *et al*. (1984) used a method where they left out the extraction step and directly transmethylated the samples with good results. Some work was also published on the combination of the extraction and transmethylation steps (Kang *et al*., 2005).

It is obvious that no matter what extraction procedure is used, great care should be taken to guard against oxidation of the extracted fatty acids. The use of an antioxidant such as butylated hydroxytoluene (BHT) must always form an integral part of this procedure. Where possible, fatty acid extracts should also be handled in an atmosphere of nitrogen (Christie, 1993).

A mixture of chloroform and methanol is probably the best general lipid extraction solution, but it is not the safest from an environmental standpoint and *n*-hexane is an extraction solution worth trying (Christie, 1993).

## 2.8 Transmethylation of fatty acids

Before the fatty acid components of any lipid can be analysed by GLC, it is necessary to convert them to low molecular weight non-polar derivatives, such as methyl esters. Although fatty acids can occur as free fatty acids in nature, they are mostly found as esters linked to a glycerol. Nearly all the important fats and oils of animal and plant origin consist almost exclusively of this simple lipid class, and are known as triacylglycerols (Figure 7) or commonly as triglycerides (Christie, 1989).

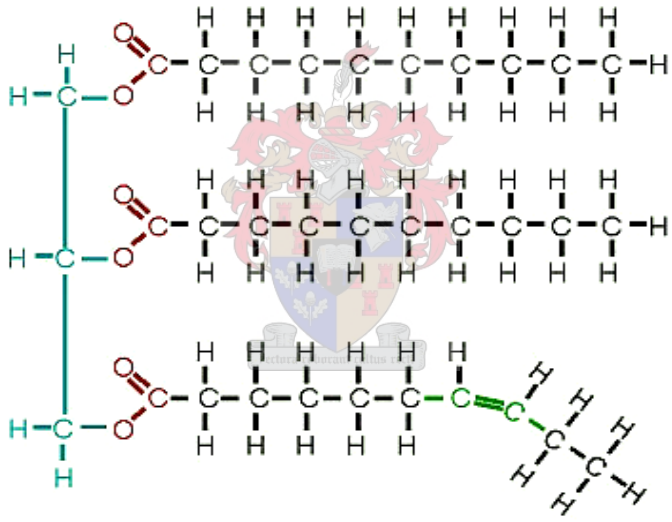


**Figure 7. The chemical structure of a triacylglycerol molecule**

The preparation of methyl esters derivatives from triacylglycerols is by far the most common chemical preparation performed by lipid analysts. In short, it means the breaking of the bond (hydrolyses) between the fatty acids and the glycerol backbone and the formation of a fatty acid methyl ester. There is no need to hydrolyse or saponify triacylglycerols to obtain free fatty acids before preparing the methyl esters, as they can be transesterified or transmethylated directly to fatty acid methyl esters (FAME) for GLC analyses (Christie, 1990). A number of different transmethylation methods have been described to form derivatives, depending on the samples and methods the analysts were using. Since this study

focuses on the identification and quantification of *cis* and *trans* fatty acids isomers in partially hydrogenated oils, only the two most common transmethylation methods for triacylglycerols will be reviewed.

### 2.8.1 Acid-catalysed transmethylation

Classic acid catalysed transesterification chemistry calls for the reaction of a triacylglycerol with alcohol in the presence of an acid catalyst to form FAME (Figure 8) for GLC analyses.

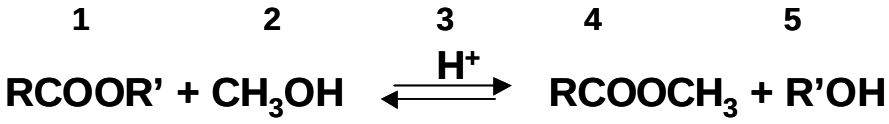



**Figure 8. Acid-catalysed transmethylation reaction of a lipid to form a methyl ester**

**1**. Lipid **2**. Methanol **3**. Acid **4**. Methyl ester **5**. Glyserol

A commonly used acid transesterification catalyst is probably boron trifluoride ($BF_3$) in methanol. This reagent could be used to transmethylate most lipid classes (Morrison *et al*., 1964). Although this reagent has serious drawbacks, it has the advantage that it can be easily purchased from a number of suppliers (Christie, 1994). By using $BF_3$ in methanol, methoxy artifacts are produced from unsaturated fatty acids by adding methanol across the double bond when high concentrations of this reagent are used (Lough, 1964). There is some evidence that the artifact formation is most likely the result of aged reagents (Fulk *et al*., 1970). The reagent has a very limited shelf life at room temperature, and if it has to be used, this should carefully be checked beforehand.

The most frequently cited reagent for the preparation of methyl esters is 5% anhydrous hydrogen chloride in methanol (Christie, 1990). In the standard transmethylation procedure, the samples were dissolved in at least a 100-fold excess of methanolic hydrogen chloride and refluxed for 2 hours or held at 50$^{o}$C overnight. Triacylglycerols are not soluble in methanolic hydrogen chloride alone, and an inert solvent, like hexane or chloroform, must be added to affect the solution before the reaction can be started. After the reaction, the methyl esters are extracted by adding water and hexane. All the different fatty acids are esterified at

approximately the same rate, so there is unlikely to be differential losses of specific fatty acids during the methylation step, except for short-chain FAME that may be lost during refluxing of the sample. Short-chain FAMEs are also soluble in water and can be lost during an aqueous extraction step. The best transmethylation methods for short-chain fatty acids are those that require no heat or aqueous extraction steps (Christopherson *et al.*, 1969).

Hydrogen chloride in methanol can be said to be the best general purpose esterifying reagent available, but its long reaction time is a disadvantage. A good alternative to hydrogen chloride in methanol is a 5% solution of concentrated sulphuric acid in methanol. This is very easy to prepare and is the preferred reagent for transmethylation of triacylglycerols (Christie, 1990). As sulphuric acid is a very strong oxidising reagent, great care must be taken when working with polyunsaturated fatty acids. However, there is no evidence of side effects when using a 5% sulphuric acid solution and moderate temperature for a short time (Christie, 1990).

### 2.8.2 Base-catalysed transmethylation

Triacylglycerols are transmethylated very rapidly in anhydrous methanol in the presence of a basic catalyst such as sodium methoxide, which facilitates the exchange between glycerol and methanol (Figure 9). The reaction is very quick and triacylglycerols are completely transesterified at room temperature in a few minutes (Marinetti, 1966).

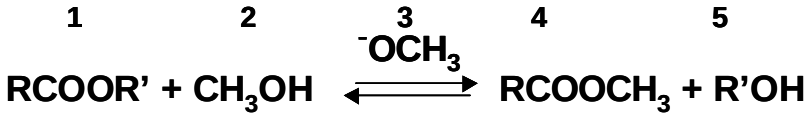



**Figure 9. Base-catalysed transmethylation reaction of a lipid to form a methyl ester**

**1**. Lipid **2**. Methanol **3**. Base **4**. Methyl ester **5**. Glycerol

The most popular basic transmethylation solvent in use is a 0.5 to 2 M sodium methoxide in anhydrous methanol. This mixture is stable for several months at refrigeration temperature if oxygen-free methanol is used for its preparation (Christie, 1972). As with acid catalysed transesterification procedures, a further solvent, such as hexane, ether or toluene is needed to solubilise the non-polar lipids after extraction. In a typical transmethylation reaction, the sample is extracted and dissolved in sufficient hexane or other solvent to get it into solution.

A 100-fold excess of 0.5 M sodium methoxide in anhydrous methanol is then added. After about 10 minutes, 0.1 ml glacial acetic acid is added to neutralise the sodium methoxide, and the methyl esters are extracted using water and hexane. Sodium methoxide in anhydrous methanol is a useful reagent for fast transmethylation of fatty acids linked by ester bonds to alcohols, but cannot form methyl esters from free fatty acids (Jamieson *et al*., 1969).

To evaluate which transmethylation method is the most suitable for the formation of FAME from margarines, two transmethylation reagents will be used: a 5% concentrated sulphuric acid in double distilled methanol solution, and a 0.5 M methoxide in anhydrous methanol solution.

## 2.9 Instrumentation

### 2.9.1 Gas liquid chromatograph

#### 2.9.1.1 Introduction

In 1901, the Russian botanist, Mikhail Tsvet, invented the first chromatography technique during his research on chlorophyll. He used liquid-adsorption columns to separate plant pigments. The technology of chromatography advanced rapidly throughout the 20$^{th}$ century, and today we have highly sophisticated instrumentation. Chromatography is a separation method that exploits the difference in partitioning behaviour of different compounds in a mixture between a mobile and a stationary phase, to separate the compounds. This involves a sample being carried in a mobile phase, normally a gas, and forced through a stationary phase that is packed into a column. The components of the sample which are to be separated have different affinities for the mobile and the stationary phases. Those components which have a higher affinity for the mobile phase will move faster through the column than those which have less affinity for the mobile phase, but more for the stationary phase. The components that have the most affinity for the stationary phase will move the slowest through the column. As a result of these differences in mobilities, sample components will become separated from each other as they travel through the column. The various components of a sample elute at different times (called retention times) resulting in separation and identification (McMurry, 2000). The distinguishing feature of GLC, is that the mobile phase is a gas, and the stationary

phase a liquid. The liquid is either coated on the wall of the column or coated onto the surface of small particles that are fixed to the wall of the column. The composition of the stationary phase generally has the greatest effect on the separation of the different components of a sample. This is because the mobile gaseous phase mainly serves as the carrier of the sample. A detection device at the end of the column records each substance as it is emitted, noting the length of time and the concentration. The length of time (retention time) identifies the substance, and the intensity of the response reveals the concentration of the substances in the sample. These two indicators are plotted on a graph (chromatogram). These chromatograms are then compared to a chromatogram of a standard mixture that is analysed under the same conditions. In this manner the components of the sample can be identified (Willet, 1987).

#### 2.9.1.2 Gas liquid chromatograph instrument

The modern gas chromatograph consists of five units (Figure 10).

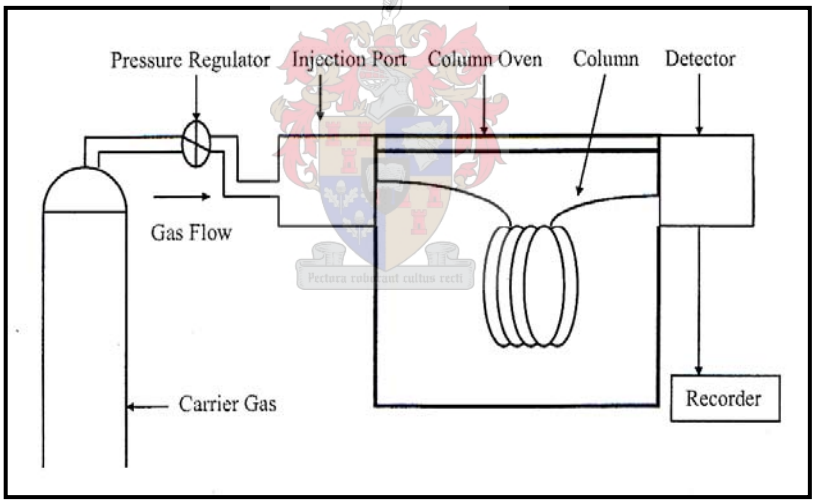



**Figure 10. Basic components of a GLC**

Courtesy: http://www.shu.ac.uk/gaschrm.htm

The gas supply unit provides all the required gas provisions to the instrument. Next is the sampling unit or the injector. Typically, the injector has its own temperature-controlling unit that monitors and controls the temperature. The oven controls the column temperature and is the most significant part of the system. The detector is located in its own oven for temperature regulation. The flame ionisation detector (FID) is the most commonly used detector in

modern GLC, because of its simplicity and reliability (Willet, 1987). The GLC system concludes with a data-processing computer, which drives the system and acquires the detectors output, processes this and prints the report. The chromatogram obtained from this procedure is always compared to the chromatogram of a standard sample run through the same instrument under the same conditions as the unknown sample.

### 2.9.1.3 Carrier gases

The type of carrier gas is important, and hydrogen is preferred to nitrogen and helium, because of its low resistance to mass transfer. Column efficiency also varies less with gas velocity over the useful working range when hydrogen, instead of nitrogen or helium is used, so that precise flow calibration is less critical (Christie, 1989). The Van Deemter plot (Figure 11) of the variation in the height of an effective theoretical plate illustrates this clearly.

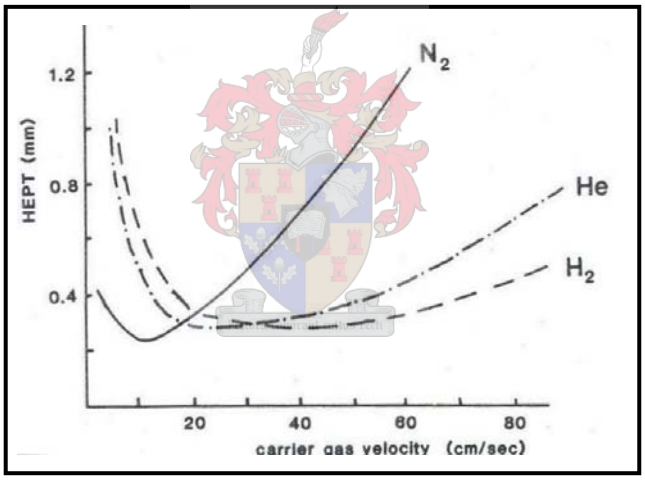



**Figure 11. Van Deemter plot indicate the effect of the velocity of nitrogen, helium and hydrogen as a carrier gas on the theoretical plate height.**

**(The lower the plate height, the better the separation)**

When the flow rate of the carrier gas is too low, there is a tendency for band broadening through longitudinal diffusion. At a too high flow rate, band broadening is diminished, but there may be insufficient time for the different components of the sample to enter into the liquid or stationary phase and thus poor separation occurs.

#### 2.9.1.4 Columns

Two general types of column are available. These are the packed and capillary columns. This study focuses on the use of capillary columns. The latest type of capillary column is made from fused silica with an internal diameter between 0.18 and 0.53 mm. They are known as wall-coated open tubular (WCOT) columns. WCOT columns consist of an open capillary tube with walls coated with a liquid stationary phase. Fused silica has proved to be an excellent medium that consists of an amorphous silicate material that is free of metal oxides and therefore very inert. They are coated on the outside with a polymeric material to prevent fractures. In the latest fused silica WCOT columns, the liquid phase is bonded chemically to the inside surface of the column, and the individual molecules of the polymeric liquid phase are cross-linked by chemical methods to improve their stability at high temperatures (Christie, 1989). The principal requirement of a liquid phase is to provide the correct degree of selectivity for the separation of the components in a sample. In lipid analyses, the main factor influencing separation is the polarity of the liquid phase (Christie, 1989). For the analyses of the different *cis* and *trans* isomers in partially hydrogenated oils, a number of different columns have been used. Most of these were very long capillary columns coated with highly polar cyanoalkyl polysiloxane stationary phases, marketed under trade names such as BPX-70 (biscyanopropylsiloxane polysilphenylene), SP-2340 (5% cyanopropyl phenyl polysiloxane + 95% bicyanopropyl polysiloxane) and CP-Sil 88 (bicyanopropyl polysiloxane) (Precht *et al*., 1996; Aro *et al*., 1998; Ball *et al*., 1993). From the literature it is clear that you need a long column with a polar stationary phase in the first place, and secondly, most experiments use isothermal column temperatures between 160$^{o}$C and 180$^{o}$C (Duchateua *et al*., 1996; Ratnayak*e et al*., 2002).

#### 2.9.1.5 Column temperature

Optimal separation of the components in the sample is greatly dependant on the column temperature. The retention time of a compound depends, not only on the type of stationary phase or the velocity of the carrier gas, but also on the column temperature. A higher column temperature will cause the compound to move faster through the column giving a shorter retention time, but this will also cause a decrease in the efficiency of the column. Although the components emerge as sharper peaks because of the increase of the vapour pressure of the

solute, the ratio of the solute in the gas phase to the liquid phase also increases. If the column temperature is too high, highly volatile components in the sample will move practically as fast as the carrier gas and will not be separated. On the other hand, at a too low column temperature, the less volatile components will hardly move through the column. Column temperature plays a critical role in the optimal separation of the different *cis* and *trans* isomers in partially hydrogenated oils. Ratnayake *et al*. (2002) found that a column temperature of 180$^{o}$C produced the fewest overlapping peaks of *cis* and *trans* isomers on 100 m SP- 2560 and CP-Sil 88 capillary columns. At this temperature all *trans* isomers, except *trans*-13 and -14, and *trans*-15, 18:1 isomers were resolved. Isothermal temperatures above and below 180$^{o}$C produced some additional overlapping (Ratnayake *et al*., 2002). Aro *et al*. (1998) also used a CP-Sil 88 capillary column, but they reported that a column temperature of 155$^{o}$C gave the best resolution of the *cis* and *trans* isomers.

No golden standard for optimal column temperature exists and each analyst must optimise the column temperature to suit his or her conditions and applications.

#### 2.9.1.6 Injectors

Samples are applied to the column by means of an injector. With the use of capillary WCOT columns, different types of injectors can be used. For optimum column efficiency, the sample should not be too large or too concentrated and should be introduced onto the column as a plug of vaporised sample. During the injection process, it is important that the sample should not change in composition and there should be no discrimination against any component in the sample. Thermal degradation or rearrangement should be negligible. No loss of column efficiency should be introduced. The solvent peak should not interfere with the detection of the solutes, and the detection time and peak areas should be reproducible. Capillary WCOT columns have a very small sample capacity, and it is relatively easy to overload a column by injecting a too large sample volume or very concentrated sample. The split injector (Figure 12) is ideal to circumvent this problem. In this study only the split injector will be used.

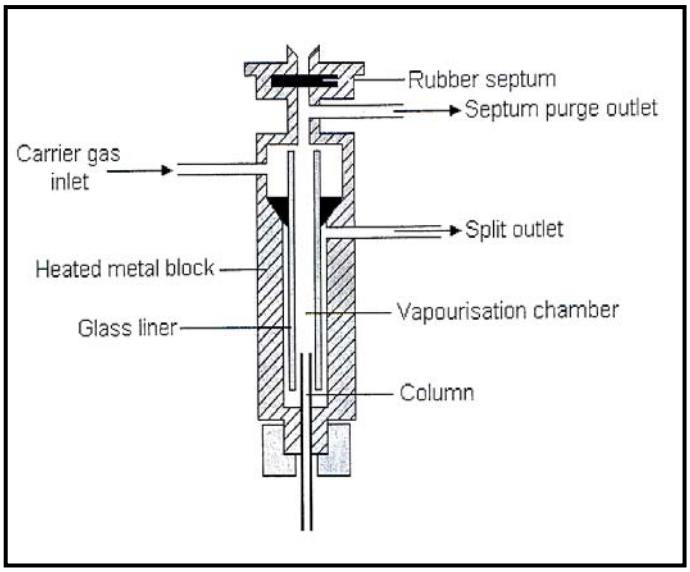



**Figure 12. Split/ Splitless injector**

Courtesy: http://www.shu.ac.uk/gaschrm.htm

The injector contains a glass liner into which the sample is injected through a septum. After injection the sample vaporises because of the high temperature of the injector and forms a mixture of carrier gas, vaporised solvent and vaporised solutes. The sample, mixed with the carrier gas, enters the injector chamber and leaves the chamber via three routes: 1) a portion purges the septum to prevent septum-bled components entering the column, and leaves via the septum purge outlet, 2) a portion flows on to the column, and 3) the largest portion of the sample exits through the split outlet.

### 2.9.1.7 Detectors

There are a large number of detectors that can be used in gas chromatography, but only a few are used to a significant extent of which the FID is the most popular (Figure 13). The FID is highly sensitive and stable, and has a low dead volume, a fast response time and is linear over a very wide concentration range. Only inert gasses and a few other volatile substances do not give a substantial signal. One great disadvantage of a FID is that the sample is destroyed because of the use of a hydrogen diffusion flame to ionise the compound for analyses. The FID responds to any molecule with a carbon-hydrogen bond. Since the FID is mass sensitive and not concentration sensitive, changes in the carrier gas flow rate have little effect on the

response of the detector. As the sample elutes from the column, it is mixed with hydrogen and passes through a flame that breaks down the molecules and produces ions. These ions carry a current that is measured by the detector, amplified and sent to the data-processing system.

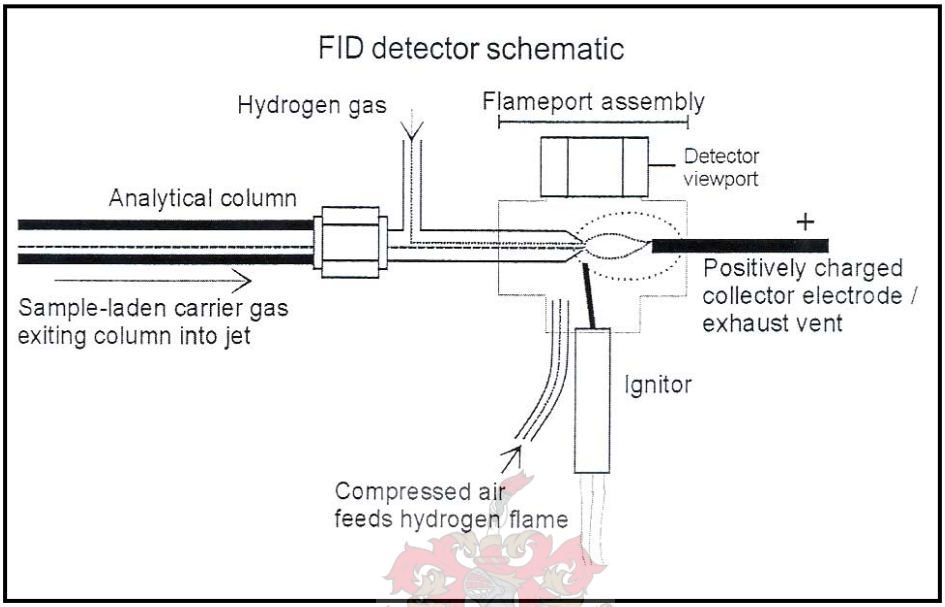



**Figure 13. Schematic drawing of a flame ionisation detector**

Courtesy: http://www.shu.ac.uk/gaschrm.htm

### 2.9.2 Capillary electrophoresis

#### 2.9.2.1 Introduction

Electrophoresis is defined as the migration of ions in an electrical field. When a positive (anode) and a negative (cathode) electrode are placed in a solution containing ions and a voltage is applied across the electrodes, the anions and the cations in the solution will move towards the electrode with opposite charge. Separation by electrophoresis relies on the speed of the mobility of the different ions in the sample. The mobility will be determined by the charge as well as the size of the ions. The higher the charge and the smaller the ion, the faster it moves.

Another very important feature of capillary electrophoresis is the flow of the buffer liquid through the capillary column that is normally made from fused silica. The surface of the inside wall of the fused silica capillary is made up of ionisable silanol groups which dissociate to produce anions ($SiO^-$), especially above a pH of 4. These groups give the wall a negative charge. When the capillary is filled with a buffer solution, the negatively charged wall will attract the positive ions in the buffer solution creating an electrical double layer and a potential difference (zeta potential) close to the capillary wall (Figure 14).

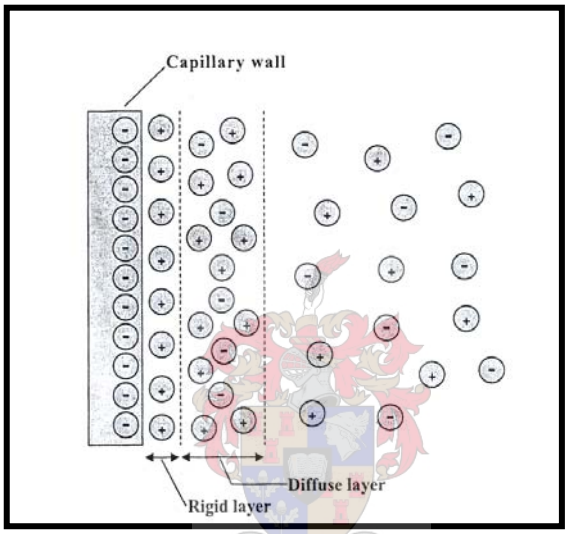



**Figure 14. Stern's model of the double-layer charge distribution at a negatively charged capillary wall leading to the generation of a Zeta potential and EOF**

When a voltage is applied across the capillary, the cations in the diffused layer will moved towards the cathode pulling with them the bulk solution in the capillary. This is called electro-osmotic flow (EOF). The charge on the capillary wall is highly dependant on the pH, which means the EOF, is also dependant on the pH, therefore, the higher the pH the greater the EOF. A benefit of EOF is its characteristic flat-flow profile, which results in sharp peaks with good resolution (Figure 15). External pump systems used in HPLC result in laminar-flow profiles with rounded broad peaks.

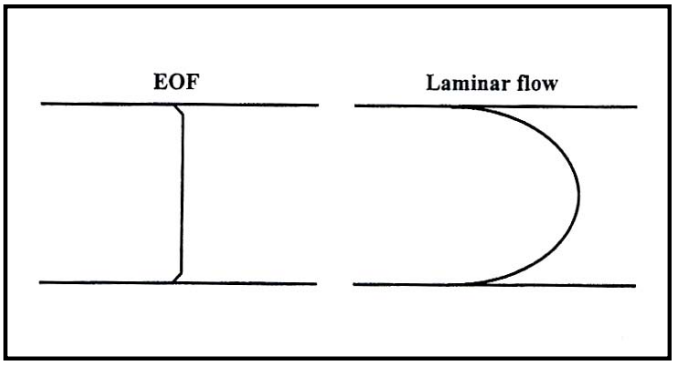



**Figure 15. Flow profiles of EOF and laminar flow**

A great advantage of EOF is that it causes migration of not only the cations, but because of the flow of the buffer, the anions and the neutral molecules will also be moved towards the cathode and the detector. The other factor affecting the mobility is the viscosity of the buffer. With the passage of an electrical current through an electrolyte buffer, heat is generated and this causes an elevation of temperature within the capillary. This heat causes a change in the viscosity of the buffer. Control of the temperature is very important as a $1^{o}C$ change in temperature can result in a 3% change in viscosity, and thus a 3% change in mobility. Temperature increase depends on the voltage applied to the system. Thus, by lowering the applied voltage, a drop in temperature can be achieved, but the theoretical equation for resolution and efficiency advocate the use of as high an electrical field as possible. A reduction in the diameter of the capillary will cause a dramatic decrease in current, this will cause a decrease in power generated, as well as in temperature. However, a reduction in the diameter of the capillary will affect the sensitivity (Heiger, 1992). A decrease in the ionic strength of the buffer can also be used to decrease the electrical current. Luckily today most of the modern instruments have a cooling facility to overcome the problem of heating.

Capillary electrophoresis comprises of a number of different operation modes that have different separation characters. Theses modes include capillary zone electrophoresis and micellar electrokinetic chromatography.

#### 2.9.2.2 Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) is probably the most commonly used CE method because of its simplicity and versatility. Samples are injected onto a narrow bore fused silica capillary (25 - 75 mm ID) and separations of the analytes are dependant upon the different migration times of the ionic species in the sample. The ends of the capillary are placed in separated buffer reservoirs and the capillary is filled with the buffer. Electrodes are positioned in the two reservoirs and connected to a high voltage power supply. Most of the time the samples are loaded onto the capillary at the anode and the detection of the analytes take place at the opposite end of the capillary. The effective mobility and therefore the separation of the analytes are dictated by their charge to mass ratio at a specific pH. However, the migration velocity of the different ions is dependant on the sum of the EOF, which is the bulk flow of the liquid in the capillary, and their respective electrophoretic mobilities. Cations with the greatest charge to mass ratio migrate first, followed by cations with a smaller ratio, then neutral molecules, followed by anions with a smaller charge to mass ratio and lastly anions with the largest charge to mass ratio.

#### 2.9.2.3 Micellar electrokinetic chromatography

Micellar electrokinetic chromatography is normally used to resolve both charged and neutral molecules in a single run. The basic principal of MEKC is the use of surfactants that are incorporated into the buffer at concentrations above the critical micelle concentration. The most commonly used surfactant is sodium dodecyl sulphate, an anionic salt. Charged micelles migrate either with or against the EOF, depending on their charge. In the case of anionic surfactants, the negatively charged head groups tend to orientate themselves on the outer surface of the micelle, with the hydrophobic tail groups orientating themselves towards the centre of the micelle. These anionic micelles are attracted to the anode, but because of the EOF moving towards the cathode, they slowly move towards the cathode. If the analytes are charged, they will migrate according to their electrophoretic mobilities, but neutral analytes will migrate with the EOF and the micelles. The more hydrophilic the neutral analyte is, the less time it will spend inside the micelle, and the quicker it will migrate with the EOF towards the cathode. On the other hand the more hydrophobic the neural analyte is, the more time it

will spend in the micelle. Extremely hydrophobic compounds will remain in the micelle and will elute with the micelle.

#### 2.9.2.4 Capillary electrophoresis instrument

The modern capillary electrophoresis instrument is a very simple design (Figure16).

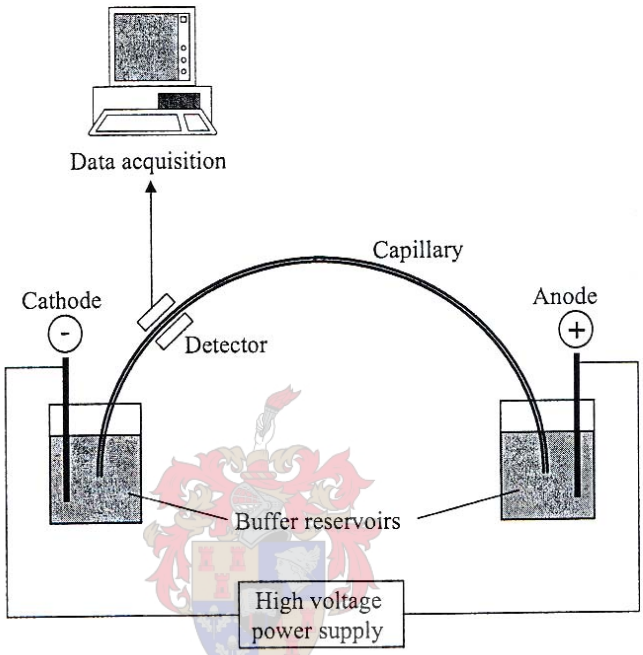



**Figure 16. Schematic representation of the arrangement of the main components of a capillary electrophoresis instrument**

The two ends of a fused silica capillary column are placed into two buffer reservoirs, each containing an electrode connected to a power supply. Samples are injected onto the capillary by putting the one end of the capillary into the sample solution and applying either an electrical potential or external pressure for a few seconds to move the sample into the capillary. After this the capillary end is put back into the buffer reservoir and an electrical potential is applied for the duration of the analyses. Detection is normally achieved through a small window, burned into the capillary, near the opposite end from where the injection took place. The most frequently used detector is a UV absorbance detector that is connected to a data processor.

#### 2.9.2.5 Capillaries

The ideal properties for a capillary would include being chemically, physically and electrically inert, as well as UV-Visible transparent, flexible, robust and inexpensive. The capillaries, which are normally used, are made from fused silica with an external cover of polyimide to give them mechanical strength, as bare fused silica is extremely fragile. A small portion of this coating is usually removed to form a window for detection purposes. This window is aligned in the optical centre of the detector. Capillaries are typically 25-100 cm long with an internal diameter of 50-75 µm. On standard commercial CE instruments, the capillary is held in a housing device to facilitate ease of capillary insertion into the instrument and to help with the temperature control of the capillary. Coating different substances onto the inner wall can also chemically modify the inner surface of the capillary. These coatings are used for a variety of purposes, such as to reduce sample absorption or to change the ionic charge of the capillary wall.

#### 2.9.2.6 Electrolyte system

The electrolyte used for a specific analysis is of critical importance as its composition determines the migration behaviour of the analytes. Because of the strong dependence of EOF and electrophoretic mobilities on the electrolyte system, careful consideration of several factors is necessary prior to the selection of a specific electrolyte system. The selected buffer should ideally possess the following properties:

- Sufficient buffering capacity for the pH working rang;
- Low absorbance at the detection wavelength;
- Temperature fluctuation must not effect its composition.

A wide range of electrolyte systems has been used to get the required separation, the majority of these being aqueous buffers. In order to perform the electrophoretic separation, the analytes must be soluble in the buffer. The ionic strength and the pH of the buffer also play an important role in the selection of the electrolytic system. Fatty acids with chain lengths of more than 14 carbons are insoluble in aqueous buffers. Thus, for the analyses of fatty acids the buffers must consist of at least some sort of organic compound.

Care must also be taken that the buffer levels in both the anodic and cathodic reservoirs remain at the same level. If the height of the buffer in the two reservoirs is not equal, a pressure difference will result and siphoning will occur, effecting migration times. Great care should be taken to restrict buffer depletion caused by electrolyses and ion migration. Buffer depletion results in pH changes, which is probably the single parameter with the greatest influence on separation. The pH of the buffer determines the electric charge of the analytes and their electrophoretic mobility, as well as the charge on the silanol groups at the capillary wall, and consequently, the EOF (Heiger, 1992).

#### 2.9.2.7 Sample introduction

The two most often-used injection methods are those of electrokinetic and hydrodynamic injection. Using the electrokinetic injection, the electrode and capillary are inserted into the sample vial and a voltage is applied for a few seconds. Field strengths about 5 times lower than that used for separation, is usually used. This method tends to have greater precision than that of the hydrodynamic technique, but it is not as reproducible. Hydrodynamic injection can be accomplished by one of two methods. Pressure can be applied at the injection end or with the application of a vacuum at the exit end of the capillary. The main advantage of hydrodynamic injection is that there is no discrimination between different sample species upon injection.



#### 2.9.2.8 Detectors

UV absorbance detectors are most frequently used.

# CHAPTER 3

# MATERIALS AND METHODS FOR GLC

## 3.1 Introduction

The complete process of fatty acid analyses by GLC consists of the extraction and transmethylation of the lipids, the injection, separation, identification and quantification of the FAME's. To achieve the required accuracy and precision, each process has to be optimised. In this Section different extraction and methylation procedures are evaluated and the procedures that give the best recovery of the extracted and transmethylated fatty acids will respectively be used to optimise the separation and final analyses of the samples. Aliquots of the samples will also be separated into their *cis* and *trans* mono-unsaturated fatty acid isomer fractions on Ag-TLC. These fractions will also be analysed by GLC to evaluate to what extent the *cis* and *trans* isomers overlapped when not pre-separated by Ag-TLC.

## 3.2 Sampling and sample handling

Eighteen different brands of hard block margarines, including brands widely used by local consumers, were bought from the local supermarket and stored at 4$^{o}$C until they could be analysed. On the day of the analyses about 100 g of each product was heated to 25$^{o}$C in an oven for 30 minutes. Each sample was thoroughly homogenised with a hand-held electric mixer. From these homogenised samples, aliquots were taken for the analyses.

## 3.3 Chemicals and gases

Analytical reagent grade chloroform, methanol and hexane (Merck Darmstadt, Germany) were re-distilled in an all glass system. All glassware was rinsed with re-distilled methanol and air-dried. All chemicals were analytical grade (Merck Darmstadt, Germany and Sigma Chemical CO. St. Louis, MO 63178 USA). The fatty acid standards were certified to be > 99% pure and purchased from Nu Chek Prep. (Nu- Chek- Prep, INC. Elysian, Minnesota,

USA). All gases employed ($N_2$, $H_2$, and medical air) were of 99.99% purity (Liquid Air, South Africa).

## 3.4 Evaluation of different lipid extraction solutions

The three extraction solutions chosen to evaluate the extraction of triacylglycerols were: a) chloroform/methanol (C:M) (2:1), b) chloroform/methanol (C:M) (1:1), and c) *n*-hexane. For the evaluation of the recovery of triacyglycerols, a test triacylglycerol sample was prepared by dissolving 102.05 mg of a > 99% pure glyceryl triheptadecanoate acid standard, (Sigma Chemical CO. St. Louis, MO 63178 USA) in 100 ml *n*-heptane. Glyceryl triheptadecanoate acid standard was used because a *trans* triacylglycerol standard was not available for the evaluation of the extraction procedure. The triacylglycerol standard that was used consisted of a glycerol with three heptadecanoic acid molecules (17:0) attached to it. Because of the structure resemblance between *trans* unsaturated fatty acids and saturated fatty acids, and the differences in structure between *cis* and *trans* unsaturated fatty acids, it was decided to use a triacylglycerol standard, formed from three saturated fatty acid molecules. The geometrical structure of the test saturated fatty acids resembled those of *trans* fatty acids.

Nine aliquots of the test sample were precisely pipetted into 50 ml extraction tubes. Lipid extracts were prepared by homogenising three of the test samples in 40 ml of C:M (2:1 v/v) containing 0.01% BHT, another three test samples in 40 ml C:M (1:1 v/v) containing 0.01% BHT, and the last three in 40 ml *n*-hexane with 0.01% BHT, by using a polytron (Kinematica, type PT 10-35, Switzerland). After homogenising, all the homogenates were filtered with sintered glass funnels. The funnels were washed with 5 ml of the different extraction solutions and the filtrates made up to 50 ml in volumetric flasks with their respective solutions. Quantitative aliquots, to give precisely 19.5 µg heptadecanoic acid (17:0), were taken from all nine volumetric flasks and FAMEs were prepared using an in-house transmethylation method based on the procedure described by Christie (1990). After cooling, 2 ml hexane and 1 ml water was added to all the samples. The solutions were thoroughly mixed on a Vortex mixer and the top hexane layers containing the FAMEs were transferred to glass tubes. The extraction procedure was repeated three times and the respective hexane phases pooled.
To each of the pooled test samples, 20.0 µg of *trans*-9, octadecenoic acid (*trans*-9, 18:1) methyl ester reference standard (Nu- Chek- Prep, INC. Elysian, Minnesota, USA) was added

as an internal standard, and the solutions were evaporated to dryness under a stream of nitrogen gas in a water bath at 40$^{o}$C. The residues were re-dissolved in 50 microliter carbon disulfide ($CS_2$) and one microliter was subjected to GLC analyses.

To verify these, by washing the samples three times with hexane, all the FAMEs were recovered from the samples; a further 2 ml of hexane was added to each of the nine sample extraction tubes and extracted again. All the hexane layers were pooled into a separate tube and evaporated to dryness. This pooled sample was analysed, with the other nine samples.

The FAMEs in all the samples were identified by GLC as described by Ball *et al*. (1993) using two Varian Model 3300 GLCs fitted with BPX-70 capillary columns. The one was fitted with a 30 m BPX-70 fused silica capillary column with an internal diameter 0.32 mm coated with 70% Cyanopropyl polysilphenylene-siloxane to a thickness of 0.25 µm (SGE International Pty Ltd, Australia). The other was fitted with a 120 m BPX 70 fused silica capillary column with an internal diameter of 0.25 mm also coated with 70% Cyanopropyl polysilphenylene-siloxane to a thickness of 0.25 µm (SGE International Pty Ltd, Australia). Both instruments were equipped with flame ionisation detectors. The analyses were done with isothermal column temperatures of 180$^{o}$C and column gas flow rate of 30 cm sec$^{-1}$. Gas flow rates were: hydrogen, 25 ml/min and air 250 ml/min. The injector temperatures were 240$^{o}$C and detector temperatures 280$^{o}$C. One microliter samples were injected manually at a split ratio of 1:80 (Ball *et al*., 1993).

## 3.5 Evaluation of lipid transmethylation procedures

For the evaluation of the best transmethylation method to be used for the preparation of FAMEs of triacylglycerols, a test sample was prepared by dissolving a > 99% pure glyceryl triheptadecanoate acid standard (Sigma Chemical CO. St. Louis, MO 63178 USA) in *n*-heptane. This test sample was extracted with the extraction solvent that gave the best recovery of the FAMEs as determined under Section 3.4. Quantitative aliquots (28.5 µg) from the extracted test sample were pipetted into six methylation tubes. To three of these, 2 ml of 5% concentrated sulphuric acid in re-distilled methanol (v/v) was added, then sealed with Teflon-lined caps and heated in a metal block for two hours at 70$^{o}$C. Thereafter they were cooled to room temperature (Christie, 1990). To the other three sample tubes, 5 ml of 0.5 M sodium

methoxide in anhydrous methanol (Aldrich Chemical CO. INC. Milwaukee, WI 53201 USA.) was added and sealed with Teflon-lined caps. These tubes were heated in a 40$^{o}$C water bath for 5 minutes, and after cooling a drop of concentrated glacial acetic acid were added to each tube to neutralise the reaction (Richardson *et al*., 1997). One millilitre of water and 2 ml of hexane were added before the tubes were vortexed for 30 seconds. Thereafter the upper hexane layers, containing the methyl esters, were transferred to extracting tubes. The extraction procedure was repeated three times and the respective hexane phases pooled. To all six tubes, 20.0 µg of *trans*-9, octadecenoic acid (*trans*-9, 18:1) methyl ester reference standard (Nu- Chek- Prep, INC. Elysian, Minnesota, USA), as an internal standard, was added and mixed well before the extracts were evaporated to dryness under a stream of nitrogen gas in a 40$^{o}$C water bath. These samples were also analysed as described under Section 3.4.

To evaluate the effect of the different sample concentrations to a constant transmethylation solution volume, three triplicate triacylglycerol test samples with concentrations of 19.5 µg/100µl, 28.5 µg/100µl and 59 µg/100µl were transmethylated with the two transmethylation solutions under investigation. After transmethylation, the samples were extracted as described earlier. To all nine tubes, 40 µg of *trans*-9, octadecenoic acid methyl ester reference standard solution was added and evaporated to dryness before subjected to GLC analyses.



## 3.6 Sample preparation

After the evaluation of the different extraction and transmethylation solutions, the methods giving the best recoveries were used for the preparation of the margarine samples for GLC analyses. The fatty acid constituents of the margarines were identified and quantified by accurately weighing 300 mg aliquots of the homogenised samples into extraction tubes, and a known concentration of glyceryl triheptadecanoate acid (17:0), as an internal standard, was added to the samples. Lipid extracts were prepared by homogenising the samples with a polytron (Kinematica, type PT 10-35, Switzerland) in 40 ml of the extraction solution that gave the best recoveries of the three solutions evaluated. After homogenising, the homogenates were filtered with sintered glass funnels. The funnels were washed with 5 ml of the extraction solution, and the filtrates made up to 50 ml with the chosen extraction solution in 50 ml volumetric flasks. Two millilitres of the transmethylation solution, which gave the

best recoveries of the two methods evaluated, were added to 500 µl of the different sample extracts, then sealed with Teflon-lined caps and transmethylated as described by Christie (1990). After cooling, 1 ml water and 2 ml hexane were added, and the samples thoroughly mixed on a Vortex mixer. The samples were extracted once only with hexane, because of the internal standard that was added to the sample before the extraction solution. The assumption can be made that if there is any loss of the sample, the same will happen to the internal standard. The top hexane layers were evaporated to dryness and re-dissolved in $CS_2$ before GLC analyses.

## 3.7 Evaluation of the two BPX-70 GLC columns

Two columns were evaluated. The one, a 30-m BPX-70 capillary column, is normally used for routine fatty acid analyses, while the other one is a 120-m BPX-70 capillary column. For the separation of the different *cis* and *trans* fatty acid isomers, most of the authors recommended a long column (Precht *et al*., 1996; Aro *et al*., 1998; Ball *et al*., 1993). A pooled margarine FAME sample was prepared for the evaluation of the two capillary columns. Two Varian Model 3300 GLCs fitted with these two columns were used for the identification of the FAMEs in the pooled sample. The one was fitted with a 30-m BPX-70 fused silica capillary column with an internal diameter of 0.32 mm and coated with 70% Cyanopropyl polysilphenylene-siloxane to a thickness of 0.25 µm (SGE International Pty Ltd, Australia). The other was fitted with a 120-m BPX 70 fused silica capillary column with an internal diameter of 0.25 mm, also coated with 70% Cyanopropyl polysilphenylene-siloxane to a thickness of 0.25 µm (SGE International Pty Ltd, Australia). Both instruments were equipped with flame ionisation detectors. The evaluation of the two columns was done with isothermal column temperatures of 180$^{o}$C and a hydrogen column flow rate of 30 cm sec$^{-1}$. Gas flow rates were: hydrogen, 25 ml/min and air 250 ml/min. The injector temperatures were 240$^{o}$C and detector temperatures 280$^{o}$C (Ball *et al*., 1993). One microlitre samples were injected manually at a split ratio of 1:80. A computer fitted with a Delta integration program (Dataworx Pty Ltd, 17/1 Goodwin St. Kangaroo Point, Brisbane, Australia.) controlled the GLC systems and did the integration of the peaks.

## 3.8 Identification of the different standard isomers

After the evaluation and selection of the column that gives the best separation of the different fatty acids in the pooled sample, the identification and elution order of the different *cis* and *trans* fatty acid isomers were done by the different retention times of *cis* and *trans* FAME standard isomers (Nu- Chek- Prep, INC. Elysian, Minnesota, USA). To further assist with the identification, Equivalent Chain-Length (ECL) values for *cis* and *trans* FAMEs from SGE Analytical Science (www.sge.com) were also used. The FAME standards were evaporated to dryness under a stream of nitrogen in a 40$^{o}$C water bath. The residues were then re-dissolved in $CS_2$ and analysed (Ball *et al.,* 1993). Different column gas flow rates between 28 and 38 cm sec$^{-1}$ and column temperatures between 151$^{o}$C and 191$^{o}$C were used to evaluate these factors on the elution order of the different standard isomers, as well as their effect on the separation power of the column.

After the selection of the best column length to use for the analyses of the margarine samples and the identification and elution order of the different standard *cis* and *trans* isomers, the pooled margarine FAME sample was injected on the chosen column. Different column temperatures between 151$^{o}$C and 197$^{o}$C and different hydrogen column flow rates between 28 and 38 cm sec$^{-1}$ were used to evaluate these effects on the separation of real margarine FAMEs, which normally has many more different fatty acids.

Aliquots of the same pooled sample were also subjected to Ag-TLC fractionation and the *cis* and *trans* mono-unsaturated FAME fractions were also subjected to GLC analyses.

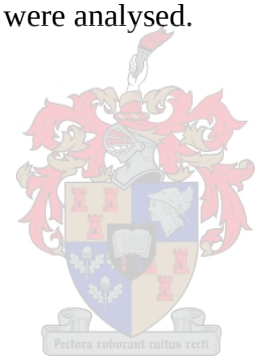
## 3.9 Evaluation of silver ion thin layer chromatography

Silver ion thin layer chromatography is use to separate the FAMEs of a sample into its saturated, *cis* mono-unsaturated, *trans* mono-unsaturated and polyunsaturated fatty acid fractions (Precht *et al.,* 1996).

Glass (20 x 20 cm) thin layer chromatography (TLC) plates (Merck, Darmstadt, Germany) coated with 0.25 mm Silica Gel 60 were dipped into a 10% (w/v) silver nitrate aqueous solution for 20 minutes, air dried and stored in a dark room. Before use, the plates were

activated at 120$^{o}$C for 30 minutes and used within one hour after cooling (Precht *et al*., 1996). Leaving a space of about 2 cm at both edges, the pooled FAME sample dissolved in *n*-heptane was applied to the plate in a narrow band. Two methyl ester standards, a *cis* and a *trans* methyl ester were also applied in the same manner. After developing with *n*-heptane/diethyl ether (90:10) in a TLC chamber lined with filter paper, the plate was air dried and the fractions were visualised by lightly spraying the plates with a 0.2% solution of 2,7-dichlorofluorescein in iso-propanol and marked under ultra violet (UV) light. The *cis* and *trans* mono-unsaturated fatty acid fractions were identified by the co-migration of the standards, scraped off separately and eluted three times with diethyl ether. The eluents were pooled and evaporated to dryness under a stream of nitrogen and re-dissolved in $CS_2$ before injecting this into the GLC.

After the optimisation of the carrier gas flow rate, the column temperature and the evaluation of the Ag-TLC results the samples were analysed.



# CHAPTER 4

# GLC RESULTS AND DISCUSSION

## 4.1 Evaluation of the different extraction solvents

Extraction is the first important step in the preparation of fatty acid methyl esters for the identification and quantification of fatty acids. The results of the different extraction solutions will be used to select an extraction solution that would give the best recovery of triacylglycerols.

The chromatograms from the GLC fitted with a 30 m BPX-70 column demonstrate the results of the triacylglycerol test sample, extracted with chloroform/methanol (C:M) (2:1) (Figure 17.1), chloroform/methanol (C:M) (1:1) (Figure 17.2) and *n*-hexane (Figure 17.3).

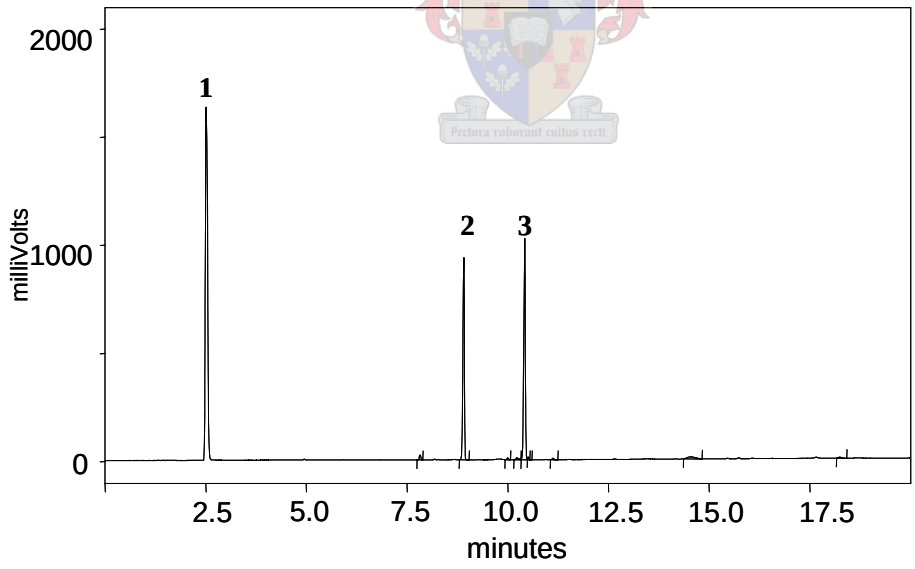



**Figure 17.1. Chromatogram of test sample and internal standard using chloroform: methanol (2:1) as the extraction solution and a 30 m BPX-70 column at 180$^{o}$C**

**Peak identification: 1= $CS_2$; 2= 17:0; 3= *trans*-9, 18:1**

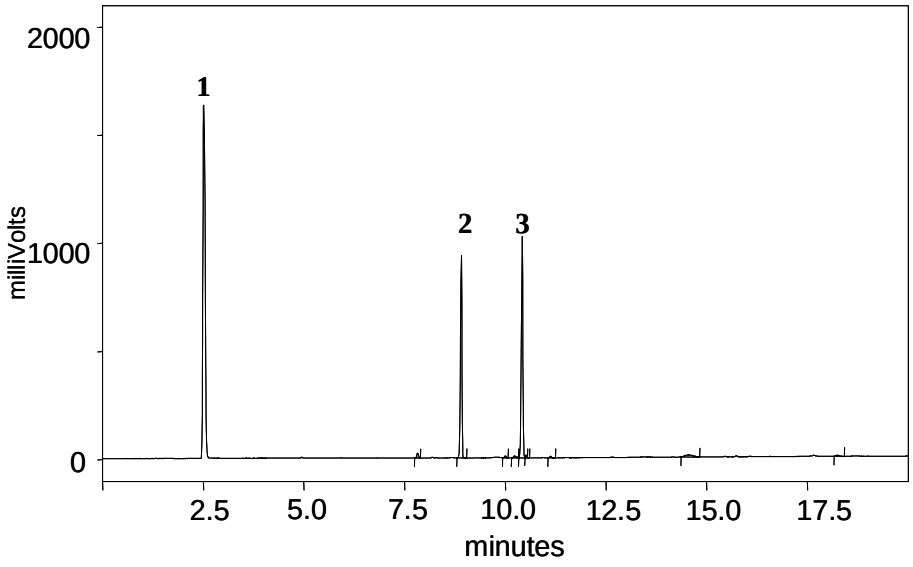



**Figure 17.2. Chromatogram of the test sample and internal standard using chloroform: methanol (1:1) as the extraction solution and a 30 m BPX-70 column at 180$^{o}$C**

**Peak identification: 1= $CS_2$; 2= 17:0; 3= *trans*-9, 18:1**

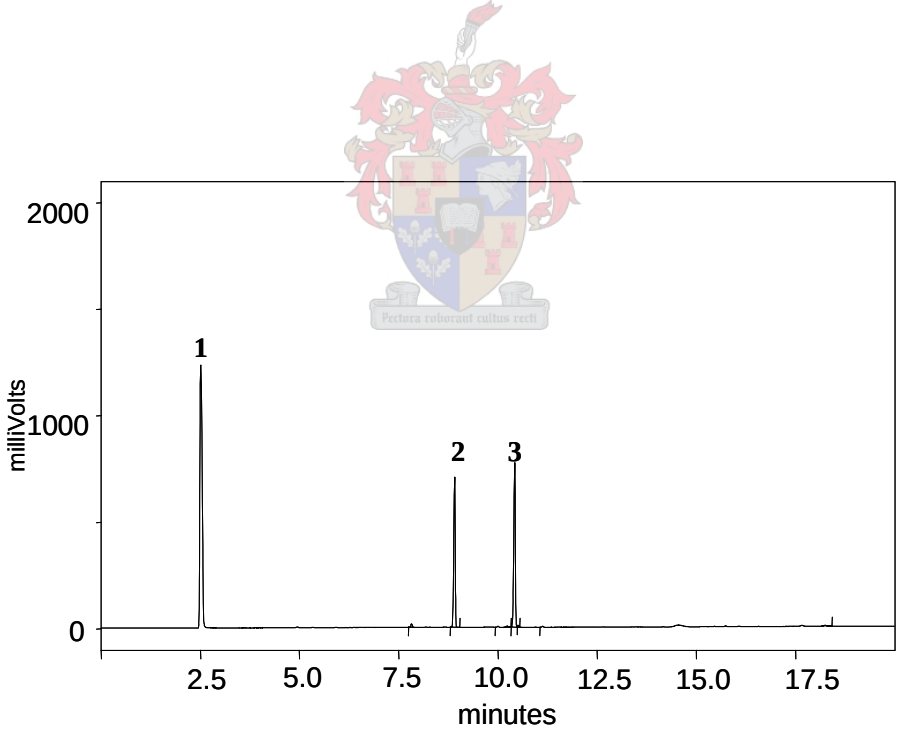



**Figure 17.3. Chromatogram of the test sample and internal standard using hexane as the extraction solution and a 30 m BPX-70 column at 180$^{o}$C**

**Peak identification: 1= $CS_2$; 2= 17:0; 3= *trans*-9, 18:1**

The peak heights of the test samples and the internal standards in all the chromatograms were less than 2000 millivolts and, therefore, well within the maximum analogue output of 10 000 millivolts of the GLCs. All measurements were also done in the linear range of the detector. The maximum range of the FID detectors is $10^7$ nano Ampere (nA), as specified by the manufacturers. For the detection of the samples the FID range was set to $10^5$ nA, well within the accepted linear range of the FID.

The two peaks representing the test sample and the internal standard using the three extraction solvents are well resolved with good baseline separation. The retention times of the two FAMEs used for the evaluation (test sample and the internal reference standard,) using the different extraction solvents were the same. The peaks were sharp with no tailing, indicating that the column was not overloaded.

The chromatograms of the three extraction methods using a 120 m BPX-70 column (Figures 17.4-17.6) show that the length of the column is an important parameter. With a 30 m BPX-70 column, the retention time of the *trans*-9, 18:1 internal standard is about 10.5 minutes, while using the same GLC conditions, but with a 120 m BPX-70 column, the retention time increases to just under 50 minutes. Thus, an increase in the column length increases the retention times.

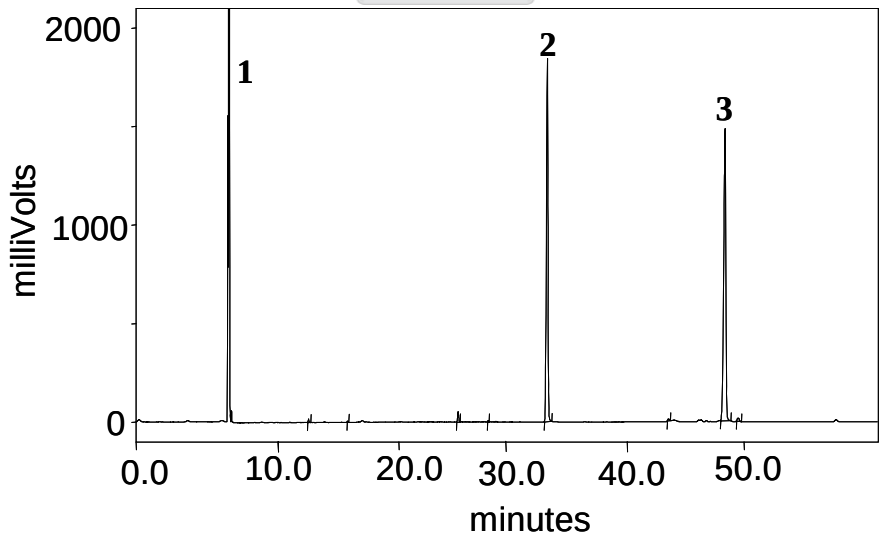



**Figure 17.4. Chromatogram of the test sample and internal standard using chloroform: methanol (2:1) as the extraction solution and a 120 m BPX-70 column at 180°C**

**Peak identification: 1= $CS_2$; 2= 17:0; 3= *trans*-9, 18:1**

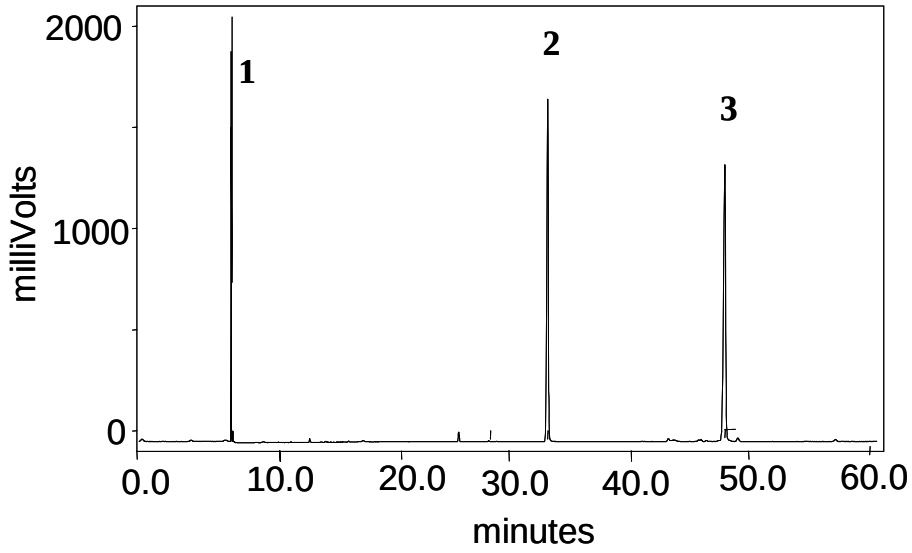



**Figure 17.5. Chromatogram of the test sample and internal standard using chloroform: methanol (1:1) as the extraction solution and a 120 m BPX-70 column at 180°C**

**Peak identification: 1= $CS_2$; 2= 17:0; 3= *trans*-9, 18:1**

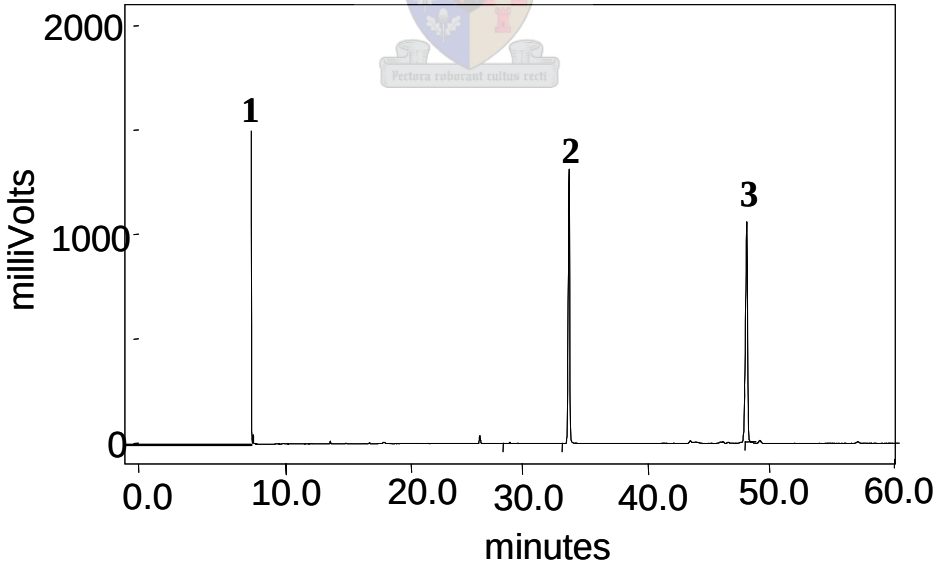



**Figure 17.6. Chromatogram of the test sample and internal standard using hexane as the extraction solution and a 120 m BPX-70 column at 180°C**

**Peak identification: 1= $CS_2$; 2= 17:0; 3= *trans*-9, 18:1**

The large difference in retention times between the 30 m and the 120 m columns is normal. The two columns used in this study were capillary columns with a small internal diameter (ID). The ID of the 30 m column was 0.32 mm and that of the 120 m column, 0.25 mm. The separation power of GLC capillary columns is given by the total chromatographic plate count. The average plate count for capillary columns of 0.32 mm ID, is about 2 500 plates/meter and for 0.25 mm ID, about 3 300 plates/meter. Thus, the longer and the smaller the internal diameter of the column, the more plates it will have and the greater the interaction of the sample components with the stationary phase. The greater the interaction, the slower the sample compounds will migrate towards the detector. Using a longer column with a small ID will retain the different compounds longer and thus extend the elution ranges of the compounds in the samples. In this way, the elution times or retention times of the different compounds are pulled apart, and a greater separation space will be available to the components in the sample eluting close together. It is well known that the different *cis* and *trans* octadecenoic acid (18:1) isomers within oils eluted in a narrow time range (Kramer *et al.*, 2004). This time range can be increased by increasing the column length as is well demonstrated by the differences in the retention times between the two peaks of the test standard and the internal standard using the two lengths. With a 30 m column the time difference between the two peaks is 2 minutes, while with the 120 m column, it is nearly 15 minutes with a column temperature of 180$^{o}$C and a hydrogen carrier gas flow rate of 30 cm sec$^{-1}$.

The sample recovery results using the three extraction methods and a 30 m BPX-70 column are summarised in Table 2, while those using a 120 m BPX-70 column are summarised in Table 3. Graph 1 graphically illustrates the results.

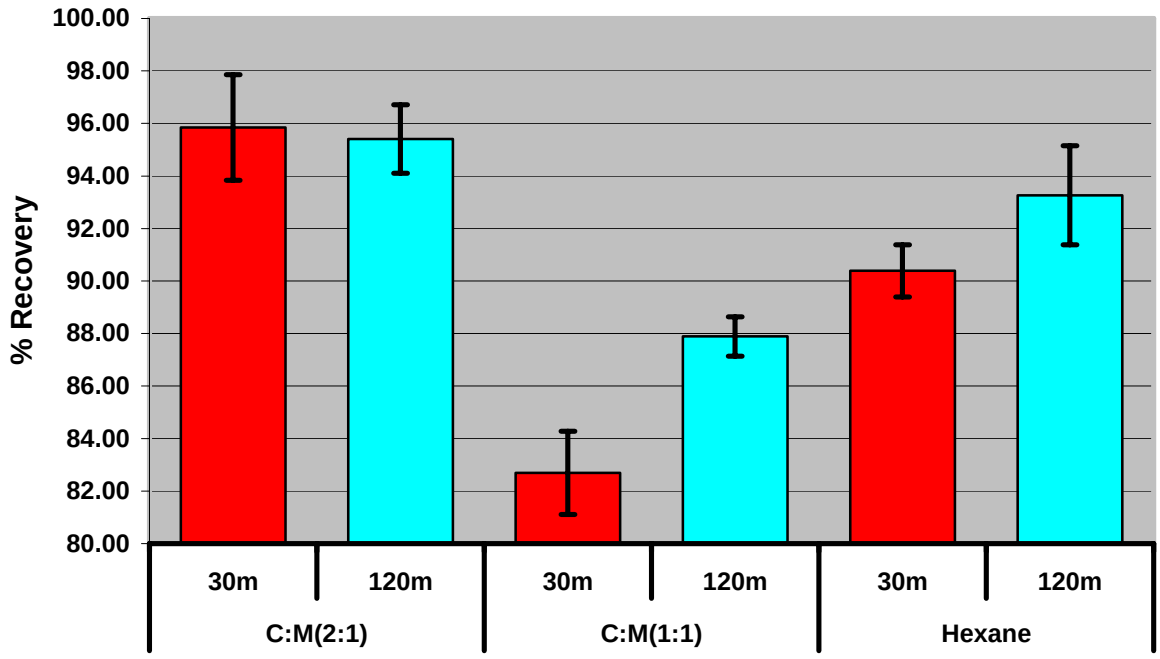
**Table 2. Recovery results of the test sample, as determined by the area counts of triplicate extractions using chloroform/methanol (2:1), chloroform/methanol (1:1) and hexane as the extraction solutions, injected into a 30 m BPX-70 column**

| Solvent | No | 17:0 | 18:1 | Avg. Rec. | STD. | %CV | True value | Measured value | Avg. | % Accuracy |
|---|---|---|---|---|---|---|---|---|---|---|
| C:M(2:1) | A | 3084084 | 3297282 | 95.8 | 2.0 | 2.1 | 19.5 | 18.7 | 19.2 | 98.5 |
| | B | 5417629 | 5590713 | | | | 19.5 | 19.4 | | |
| | C | 3782215 | 3894780 | | | | 19.5 | 19.4 | | |
| C:M(1:1) | A | 2483916 | 2943096 | 82.7 | 1.6 | 1.9 | 19.5 | 16.9 | 16.5 | 84.6 |
| | B | 2721154 | 3302212 | | | | 19.5 | 16.5 | | |
| | C | 3006318 | 3698870 | | | | 19.5 | 16.3 | | |
| Hexane | A | 3122132 | 3435303 | 90.4 | 1.0 | 1.1 | 19.5 | 18.2 | 18.1 | 92.8 |
| | B | 2593123 | 2905583 | | | | 19.5 | 17.8 | | |
| | C | 4143872 | 4552043 | | | | 19.5 | 18.2 | | |



**Table 3. Recovery results of the test sample, as determined by the area counts of triplicate extractions using chloroform/methanol (2:1), chloroform/methanol (1:1) and hexane as the extraction solutions, injected into a 120 m BPX-70 column**

| Solvent | No | 17:0 | 18:1 | Avg. Rec. | STD. | %CV | True value | Measured value | Avg. | % Accuracy |
|---|---|---|---|---|---|---|---|---|---|---|
| C:M(2:1) | A | 13081019 | 13786297 | 95.4 | 1.3 | 1.4 | 19.5 | 19.0 | 19.1 | 97.9 |
| | B | 15940621 | 16876832 | | | | 19.5 | 18.9 | | |
| | C | 21377090 | 22061821 | | | | 19.5 | 19.4 | | |
| C:M(1:1) | A | 16636799 | 19117020 | 87.9 | 0.7 | 0.8 | 19.5 | 17.4 | 17.6 | 90.2 |
| | B | 18745484 | 21225704 | | | | 19.5 | 17.7 | | |
| | C | 22846331 | 25867776 | | | | 19.5 | 17.7 | | |
| Hexane | A | 18831374 | 20657706 | 93.3 | 1.9 | 2.0 | 19.5 | 18.2 | 18.7 | 95.9 |
| | B | 37004800 | 39031132 | | | | 19.5 | 19.0 | | |
| | C | 28727594 | 30614273 | | | | 19.5 | 18.8 | | |




**Graph 1. The percentage recovery of the test sample using a 30 m and a 120 m BPX-70 capillary column and chloroform:methanol (2:1), chloroform:methanol (1:1) and hexane as the extraction solutions**

With a 30 m column, the average recovery using C:M (2:1) was 95.8 ± 2.0%, C:M (1:1) was 82.7 ± 1.6%, and with hexane it was 90.4 ± 1.0%. With the 120 m column, the average recovery was 95.4 ± 1.3%, with C:M (2:1), 87.9 ± 0.7%, with C:M (1:1) and with hexane it was 93.3 ± 1.9%. The coefficient of variants of all three methods using both columns was below 5%. Of the three extraction methods, the accuracy using the C:M (2:1) extraction solution, on both columns, was the nearest to 100 %. The differences in sample recovery as illustrated in Graph 1, between a 30 m and 120 m column, using the same extraction solution, cannot be explained. Graph 1 also illustrates that the C:M (2:1) extraction solution gave the best recovery, despite the column length, while C:M (1:1) gave the lowest average recovery on both columns. This can be because of the chloroform/methanol ratio difference between the two extraction solutions. For the identification and quantification of individual fatty acids without isomers, a 30 m column gives good results in a short analytical time. The retention time of the *trans*-9, 18:1 isomer, used as an internal reference standard was less than 11 minutes using a 30 m column, while it increased to 50 minutes with a 120 m column. This longer analytical time will have a negative effect on the number of samples that can be analysed during a working day. On the other hand, a longer column with more theoretical plates will retain the different compounds longer and thus extend the elution ranges of the

compounds in the samples. In this way, the retention times of the different compounds are pulled apart and a greater separation space is available for the fatty acid isomers eluting closely together, thereby contributing to less overlapping of the peaks.

The chromatogram in Figure 17.7 shows the results of the final pooled hexane phases. The re-extracted residues yielded no detectable peaks proving that all the FAMEs were extracted during the first three hexane extractions. By doing this confirms that washing the samples with hexane three times, extracts all the FAMEs. However, this does not guarantee that all the fatty acids are transmethylated.

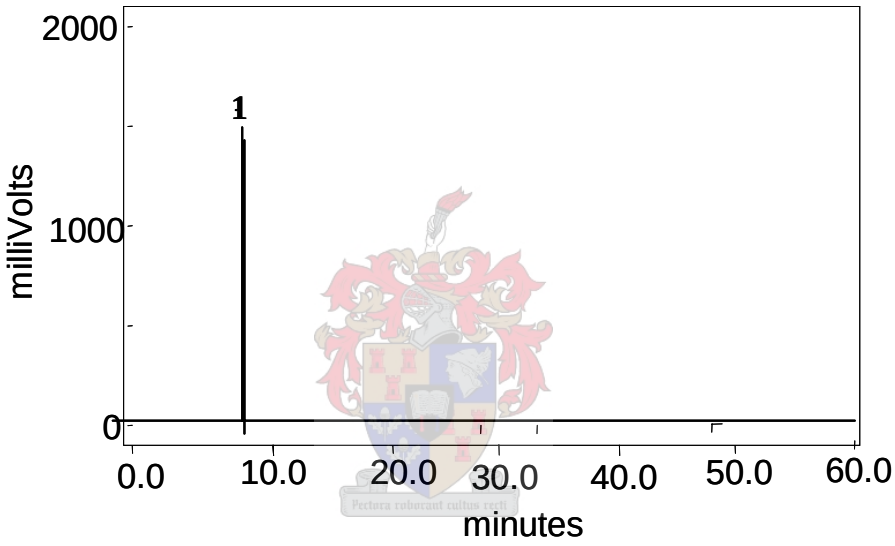



**Figure 17.7. The chromatogram of the final pooled hexane extractions to verify that washing the samples three times with hexane recovered all FAME in the nine test samples**

**Peak identification: 1= Solvent peak**

Based on the improved test sample recoveries using C:M (2:1, v/v) with both column lengths, it was decided to use C:M (2:1, v/v) as the preferred solution for the extraction of the triacylglycerols in the margarine samples.

## 4.2 Evaluation of the two transmethylation solvents

The evaluation of the different extraction solutions showed that C:M (2:1) gave the best recovery of the test sample and for the evaluation of the two transmethylation solvents the test samples were extracted with C:M (2:1).

The chromatograms (Figures 18.1-18.4) that were generated when using 30 m and 120 m BPX-70 capillary columns with the two transmethylation reagents, showed that the peaks of the test sample and the internal FAME reference standard were well resolved and sharp. The retention times of the two peaks using a 30 m column were the same for the two transmethylation reagents. The same observation was made using a 120 m column, except that the retention times were obviously longer.

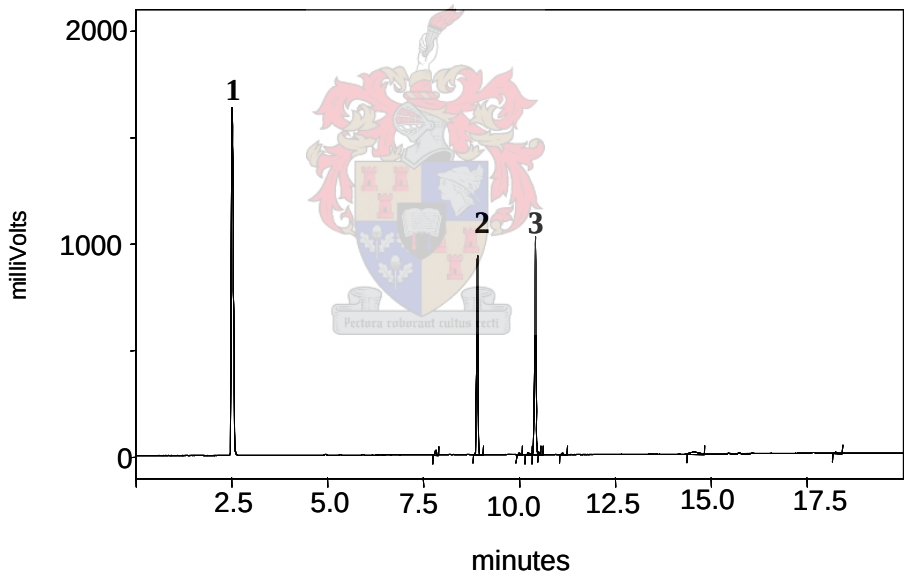



**Figure 18.1. Chromatogram of the test sample using 5% concentrated sulphuric acid in methanol as the transmethylation reagent with a 30 m BPX-70 column at 180$^{o}$C**

**Peak identification: 1= $CS_2$; 2= 17:0; 3= *trans*-9, 18:1**

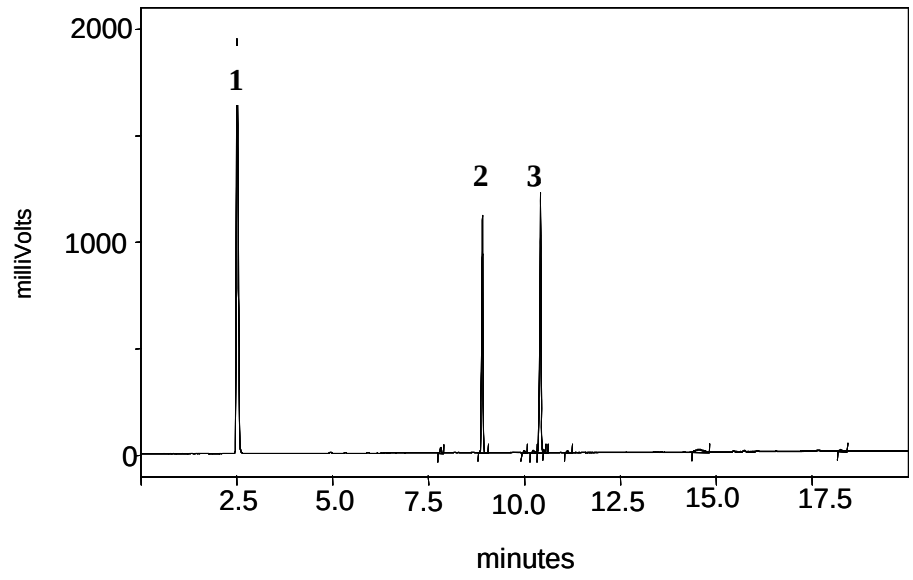



**Figure 18.2. Chromatogram of the test sample using 0.5 M methoxide as the transmethylation reagent, with a 30 m BPX-70 column at 180°C**

**Peak identification: 1= $CS_2$; 2= 17:0; 3= *trans*-9, 18:1**

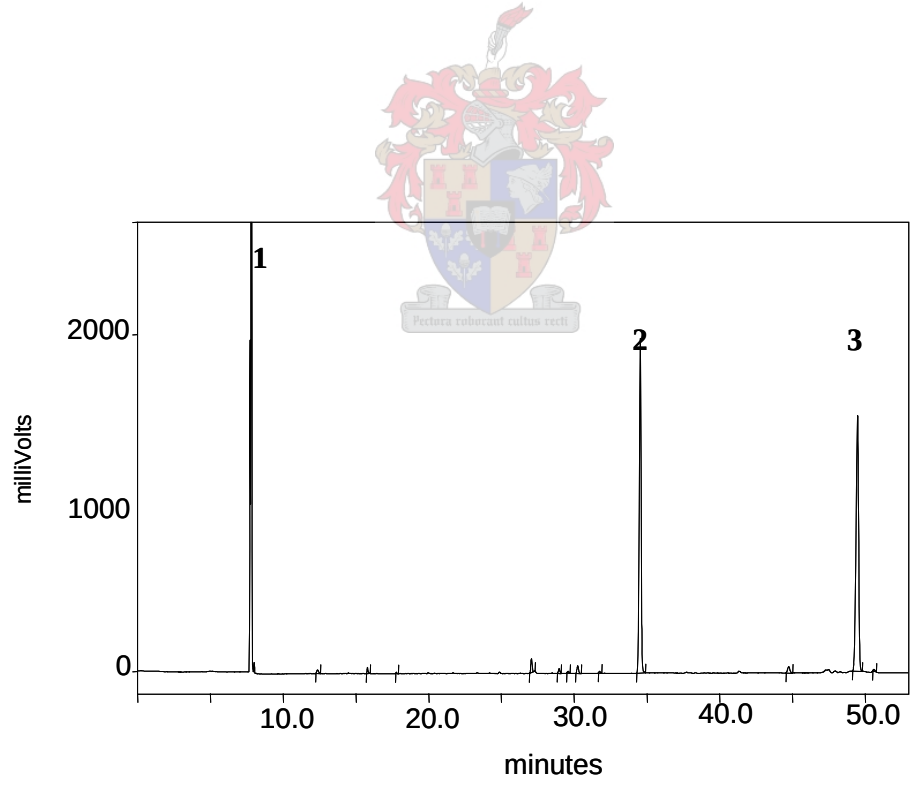



**Figure 18.3. Chromatogram of the test sample using 5% concentrated sulphuric acid in methanol as the transmethylation reagent with a 120 m BPX-70 column at 180°C**

**Peak identification: 1= $CS_2$; 2=17:0; 3= *trans*-9, 18:1**

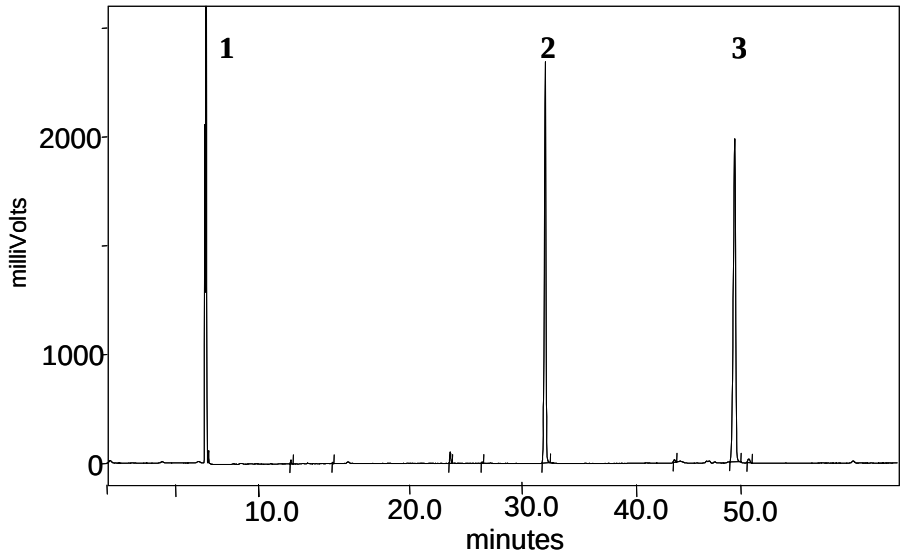



**Figure 18.4. Chromatogram of the test sample using 0.5 M methoxide as the transmethylation reagent with a 120 m BPX-70 column at 180°C**

**Peak identification: 1= $CS_2$; 2= 17:0; 3= *trans*-9, 18:1**

The sample recovery results using the two transmethylation methods and a 30 m BPX-70 column are summarised in Tables 4 and the results using a 120 m BPX-70 column are summarised in Table 5.

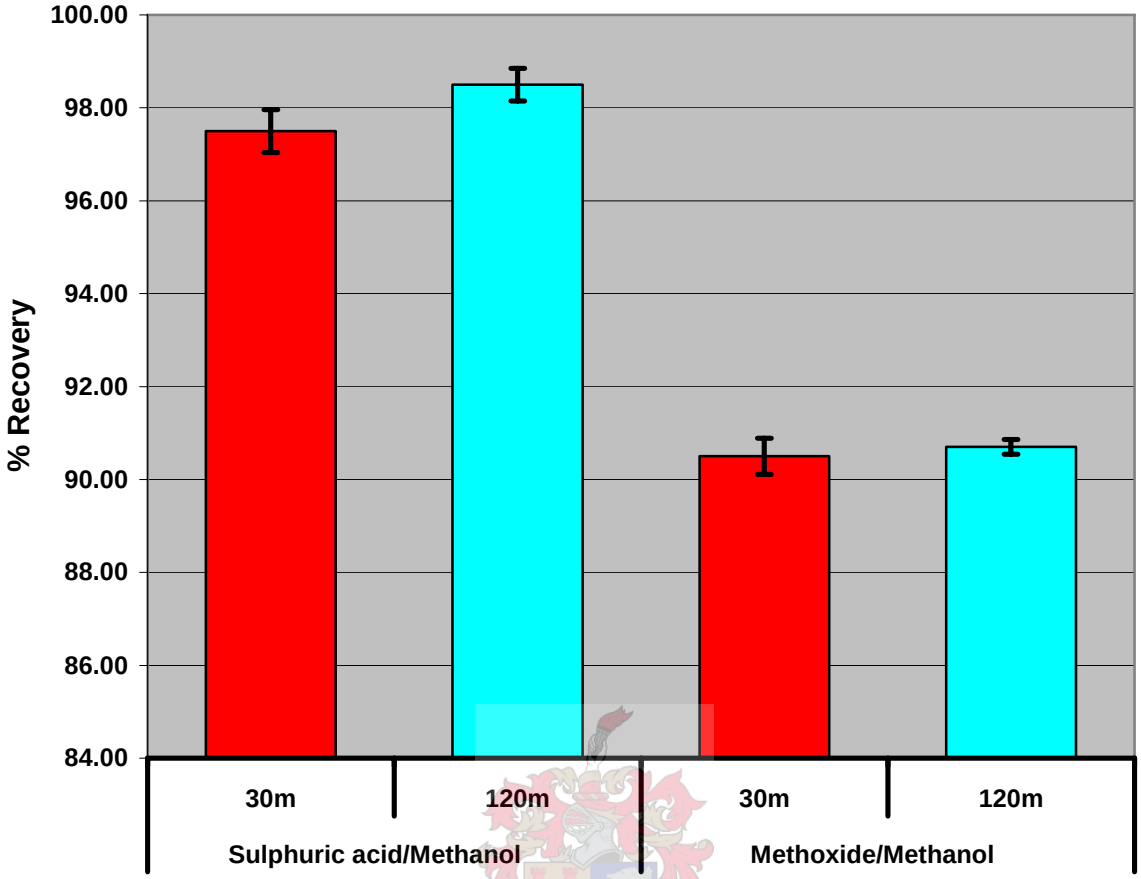
**Table 4. Recovery results as determined by the GLC area counts of triplicate extractions when using 5% sulphuric acid/methanol and 0.5 M sodium methoxide/methanol transmethylation solvents and a 30 m BPX-70 column**

| Method | No | 17:0 | 18:1 | Avg. Rec. | Std | %CV | True Value | Measured Value | Avg. | % Accuracy |
|---|---|---|---|---|---|---|---|---|---|---|
| $H_2SO_4$ | A | 5602292 | 4005386 | 97.5 | 0.5 | 0.5 | 28.5 | 28.0 | 27.8 | 97.5 |
| | B | 4476612 | 3284426 | | | | 28.5 | 27.3 | | |
| | C | 4833101 | 3436195 | | | | 28.5 | 28.1 | | |
| | | | | | | | | | | |
| $NaOCH_3$ | A | 3500466 | 2702509 | 90.5 | 0.4 | 0.4 | 28.5 | 25.9 | 25.8 | 90.5 |
| | B | 2618442 | 2003865 | | | | 28.5 | 26.1 | | |
| | C | 4020185 | 3167891 | | | | 28.5 | 25.4 | | |

**Table 5. Recovery results as determined by the GLC area counts of triplicate extractions when using 5% sulphuric acid/methanol and 0.5 M sodium methoxide/methanol transmethylation solvents and a 120 m BPX-70 column**

| Method | No | 17:0 | 18:1 | Avg. Rec. | Std | %CV | True Value | Measured Value | Avg. | % Accuracy |
|---|---|---|---|---|---|---|---|---|---|---|
| $H_2SO_4$ | A | 28118396 | 19811446 | 98.5 | 0.4 | 0.4 | 28.5 | 28.4 | 28.0 | 98.2 |
| | B | 30165464 | 21713654 | | | | 28.5 | 27.8 | | |
| | C | 32183814 | 23183919 | | | | 28.5 | 27.8 | | |
| | | | | | | | | | | |
| $NaOCH_3$ | A | 24791918 | 19283408 | 90.7 | 0.2 | 0.2 | 28.5 | 25.7 | 25.8 | 90.5 |
| | B | 23882741 | 18372506 | | | | 28.5 | 26.0 | | |
| | C | 28765312 | 22346517 | | | | 28.5 | 25.7 | | |

With a 30 m column, the average recovery was 97.5 ± 0.5% and on a 120 m column it was 98.5 ± 0.4% using 5% concentrated sulphuric acid in methanol. With 0.5 M sodium methoxide in methanol the recovery was 90.5 ± 0.4% and 90.7 ± 0.2% on a 30 m and a 120 m column, respectively. These results showed that 5% concentrated sulphuric acid in methanol gave a ± 7% better sample recovery than 0.5 M sodium methoxide in methanol for both the column lengths. The % accuracy of the 5% sulphuric acid/methanol transmethylation reagent varied between 97.5% and 98.2% using a 30 m and a 120 m column, respectively. With 0.5 M sodium methoxide in methanol the accuracy was 90.5% for both columns. These results illustrated that there is some sample lost when using 0.5 M sodium methoxide in methanol as the transmethylation reagent, and that the same loss was noticed using both column lengths. It was clearly shown that 5% concentrated sulphuric acid in methanol, as a transmethylation solution, gave the least biased results and a slightly better sample recovery on both columns.




**Graph 2. The percentage recovery of the test sample using 5% sulphuric acid/ methanol and 0.5 M sodium methoxide/methanol transmethylation reagents after analysis with two GLCs equipped with 30 m and 120 m BPX-70 columns**

Graph 2 shows the recoveries using 5% concentrated sulphuric acid in redistilled methanol as the transmethylation reagent compared to 0.5 M sodium methoxide/methanol . For the routine analyses of FAMEs a 30 m column is preferred considering the shorter analytical time, though it will have to be tested whether a short column will have enough separation power to identify and quantify the different *cis* and *trans* fatty acid isomers in a partially hydrogenated oil sample. These isomers normally elute before and after *cis*-9, 18:1. The same graph shows that a 5% concentrated sulphuric acid in redistilled methanol, as the transmethylation reagent, gives ± 8% better test sample recovery than 0.5 M sodium methoxide/methanol on both columns.

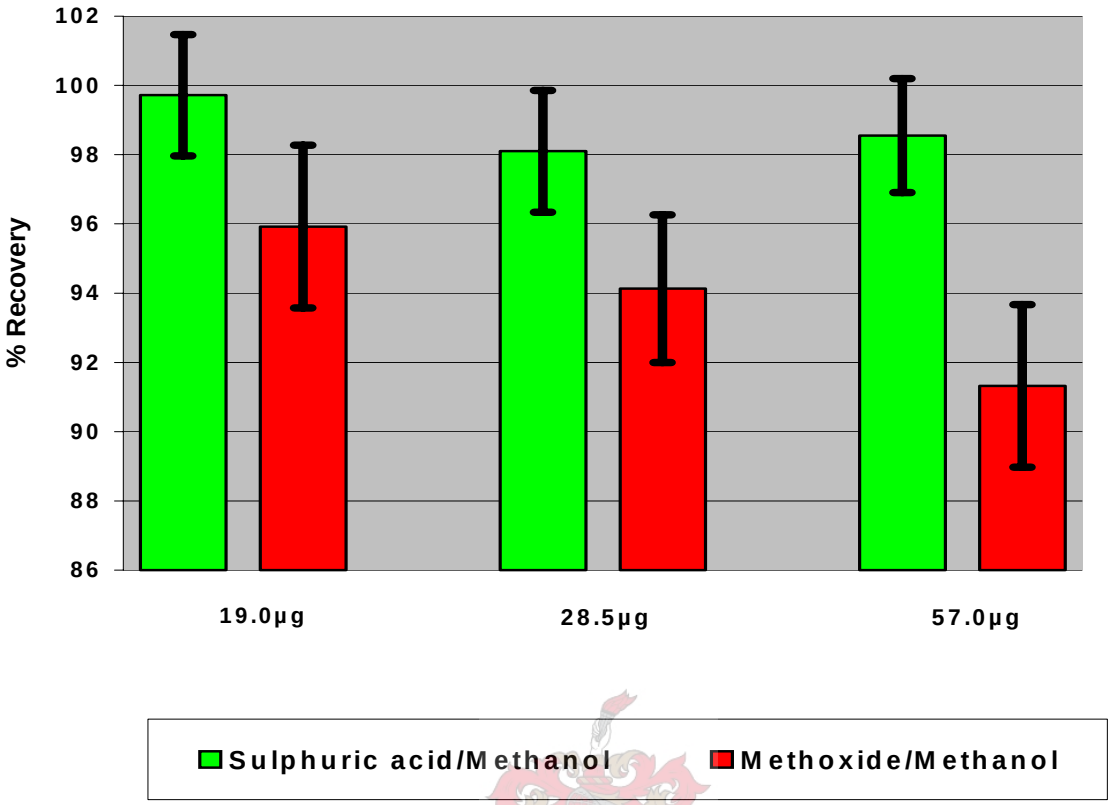
Because of the better recovery results generated by the 120 m column when using the two transmethylation reagents, only the 120 m BPX-70 column was used to evaluate the ratio effect of different sample concentrations to fixed volumes of the two transmethylation reagents.

The results in Table 6 show that with a sample containing 19.0 µg triacylglycerol, the recovery was 99.7 ± 1.7% with 5% sulphuric acid/ methanol and 95.9 ± 2.3% with 0.5 M sodium methoxide/ methanol. However, with a sample containing 28.5 µg triacylglycerol, the recoveries dropped to 98.1 ± 1.8% and 94.1 ± 2.1%, respectively. With a higher sample concentration, the effect was even more pronounced. With a sample containing 57.0 µg triacylglycerol, the recovery was 98.5 ± 1.6% using 5% sulphuric acid/ methanol, and with 0.5 M sodium methoxide/ methanol the recovery dropped to 91.3 ± 1.1%.

These results demonstrated that the recovery of samples with concentrations ranging from 19.0 µg to 57 µg, transmethylated with 2 ml of 5% concentrated sulphuric acid/ methanol reagent, is almost 100%. With 5 ml of 0.5 M sodium methoxide/ methanol solution, the recovery dropped from 95.9 ± 2.3% to just more than 90% with increasing sample concentrations. This demonstrated that the ratio between the sample triacylglycerols concentration and the volume of the transmethylation reagent had a notable effect on the recovery of the samples when using 0.5 M sodium methoxide/ methanol as the transmethylation solution.

**Table 6. Recovery results of different test samples concentrations, using 5% sulphuric acid/ methanol and 0.5 M sodium methoxide/ methanol reagents as the two transmethylation reagents**

| Method | Conc. | No | 17:0 | 18:1 | Rec. | % Rec. | Avg. | STD | %CV | Avg. Rec. | % Accuracy |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $H_2SO_4$ | 57.0 | A | 28118396 | 19811446 | 56.8 | 99.6 | 98.5 | 1.6 | 1.7 | 56.2 | 98.6 |
| | | B | 36781234 | 25968367 | 56.7 | 99.4 | | | | | |
| | | C | 67342156 | 48894532 | 55.1 | 96.7 | | | | | |
| | | | | | | | | | | | |
| $H_2SO_4$ | 28.5 | A | 8867279 | 12671829 | 28.0 | 98.2 | 98.1 | 1.8 | 1.8 | 28.0 | 98.2 |
| | | B | 10908743 | 15342318 | 28.4 | 99.8 | | | | | |
| | | C | 20132314 | 29345389 | 27.4 | 96.3 | | | | | |
| | | | | | | | | | | | |
| $H_2SO_4$ | 19.0 | A | 9566304 | 19887654 | 19.2 | 101.3 | 99.7 | 1.7 | 1.8 | 18.9 | 99.5 |
| | | B | 15432314 | 33213156 | 18.6 | 97.8 | | | | | |
| | | C | 12349548 | 25983210 | 19.0 | 100.1 | | | | | |
| | | | | | | | | | | | |
| $NaOCH_3$ | 57.0 | A | 24791918 | 19283408 | 51.4 | 90.2 | 91.3 | 1.1 | 1.2 | 52.1 | 91.4 |
| | | B | 17654321 | 13564309 | 52.1 | 91.3 | | | | | |
| | | C | 32345821 | 24567892 | 52.7 | 92.4 | | | | | |
| | | | | | | | | | | | |
| $NaOCH_3$ | 28.5 | A | 6475466 | 9875356 | 26.2 | 92.0 | 94.1 | 2.1 | 2.3 | 26.8 | 94.0 |
| | | B | 13498760 | 19675438 | 27.4 | 96.3 | | | | | |
| | | C | 29874523 | 44567234 | 26.8 | 94.1 | | | | | |
| | | | | | | | | | | | |
| $NaOCH_3$ | 19.0 | A | 6380346 | 14153521 | 18.0 | 94.9 | 95.9 | 2.3 | 2.4 | 18.2 | 95.8 |
| | | B | 10987694 | 23458712 | 18.7 | 98.6 | | | | | |
| | | C | 21348125 | 47685432 | 17.9 | 94.2 | | | | | |




**Graph 3. The percentage recoveries of different samples, using 5% sulphuric acid/ methanol and 0.5 M sodium methoxide/ methanol transmethylation reagents**

Graph 3 graphically illustrates the concentration effect. The effect is not so drastic using 5% sulphuric acid/ methanol, but with 0.5 M sodium methoxide/ methanol there is a linear drop in sample recoveries, with an increase in sample concentration. From these results, it is clear that 5% sulphuric acid/methanol transmethylation reagent would be the better choice when working with samples containing an unknown fatty acid concentration. Although 0.5 M sodium methoxide/methanol transmethylate triacylglycerols rapidly, a loss of FAMEs occurred in samples with high triacylglycerols concentrations.

Based on the ± 8% better test sample recovery, as well as the nearly 100% recovery of samples with different FAME concentrations, it was decided to use 5% concentrated sulphuric acid in redistilled methanol (v/v) as the transmethylation reagent for the preparation of FAMEs from the margarine samples.

## 4.3 Evaluation of the two columns

Although a 30 m BPX 70 column gave good separations with a short analysis time of the normal occurring fatty acids, samples like partially hydrogenated oils with a large number of different isomers, especially those occurring between 18:0 and 18:2, could not be separated completely. This observation is supported by Kramer *et al.* (2004). The overlapping of the different *cis* and *trans* 18:1 fatty acid isomers are illustrated in the chromatogram of the pooled margarine sample in Figure19.1.

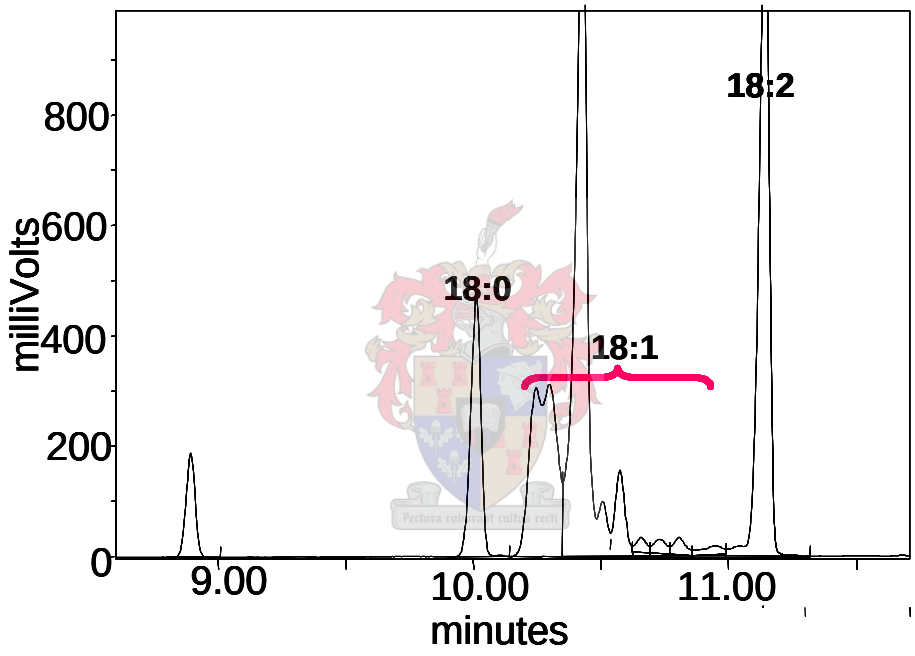



**Figure 19.1. Part of a chromatogram showing the different 18:1 fatty acid isomers (under the bracket) using a 30 m BPX 70 column at 180°C**

From Figure 19.1 it is evident that some isomers are overlapping when using a 30 m BPX-70 column. Even the use of different column temperatures and column gas flow rates, did not improve the separation. The analytical elution range of the different *cis* and *trans* 18:1 isomers are contracted into a too narrow retention time range for proper baseline separation. The 120 m BPX-70 column with a smaller ID and about 220 000 theoretical plates, compared to the 80 000 theoretical plates for a 30 m column (as specified by the manufacturer) provided

the required mechanism for extending the retention times of the different fatty acid isomers by retaining the different compounds longer. In this way, the retention times of the different isomers were pulled apart and a greater separation space became available to the different isomers, allowing more isomers to be identified (see Figure 19.2). A disadvantage of using longer columns was the longer analysis time per sample. However, this longer analytical time increased the number of isomers that could be identified and quantified.

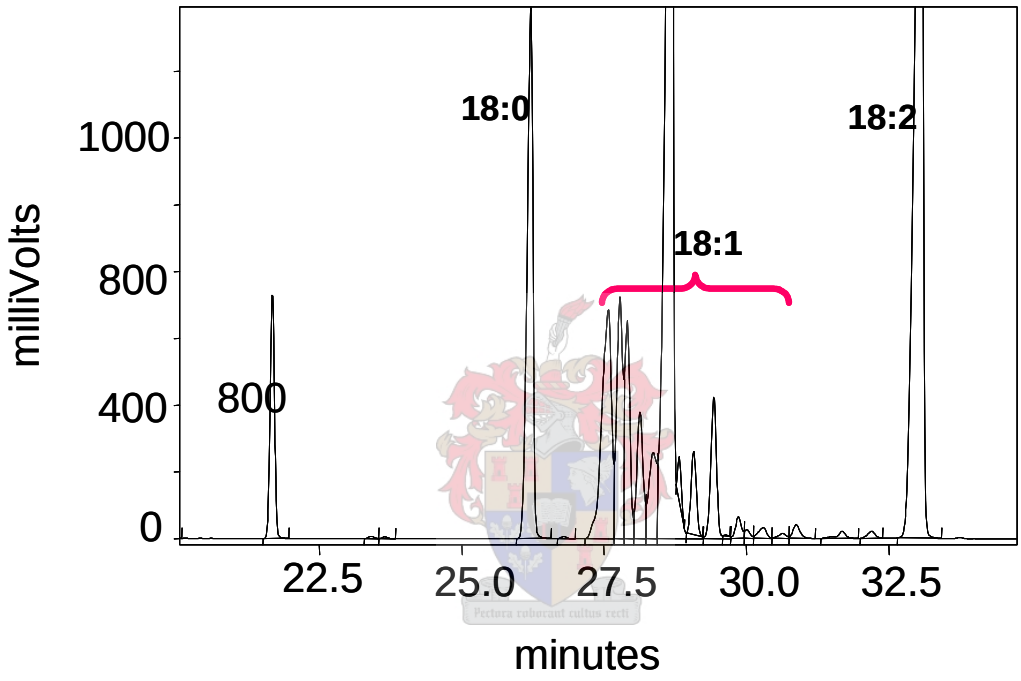



**Figure 19.2. Part of a chromatogram showing the different 18:1 fatty acid isomers (under the bracket) using a 120 m BPX 70 column at 180$^{o}$C**

Based on the inability of a 30 m BPX-70 column to separate most of the different *cis* and *trans* 18:1 isomers, it was decided only to use a 120 m BPX-70 capillary column for the identification and quantification of the different normal occurring fatty acids, as well as the different *cis* and *trans* 18:1 isomers in the margarine samples.

## 4.4 Identification of the standard isomers

The GLC chromatograms (Figures 20.1-20.4) of the standard *cis* and *trans* FAME mixture, using a 120 m BPX-70 capillary column, show the elution order to be as follows: *trans*-6, *trans*-9 and *trans*-11 followed by *cis*-6, *cis*-9 and *cis*-11. The use of different column temperatures between 151$^{o}$C and 191$^{o}$C and different hydrogen column gas flow velocities has no effect on the elution order of the different isomers in the standard mixture. This was confirmed in a technical article by SGE on the analyses of 18:1 positional isomers using a 120 m BPX-70 capillary column (www.sge.com).

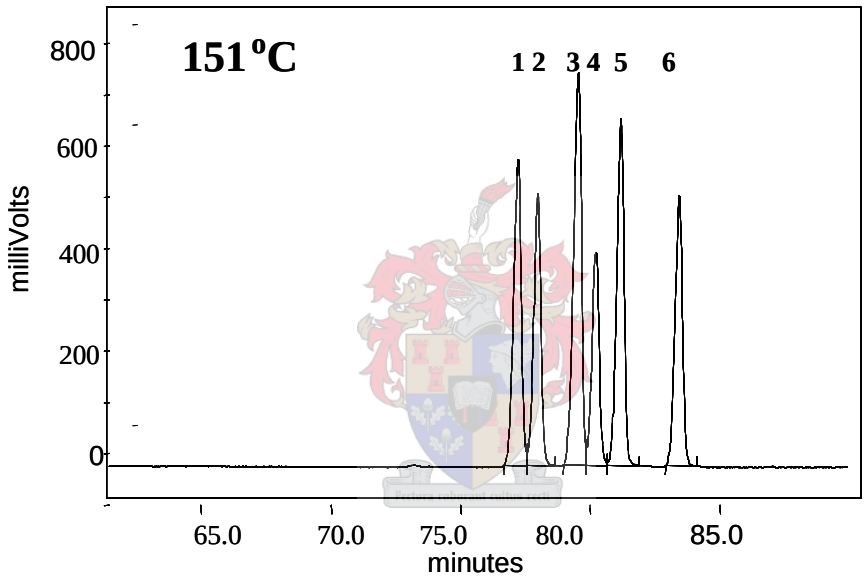



**Figure 20.1. Part of the chromatogram showing the separation of six standard 18:1 isomers analysed at a column temperature of 151$^{o}$C on a 120 m BPX-70 capillary column**

**Peak identification: 1= t6, 2= t9, 3= t11, 4=c6, 5=c9, 6=c11**

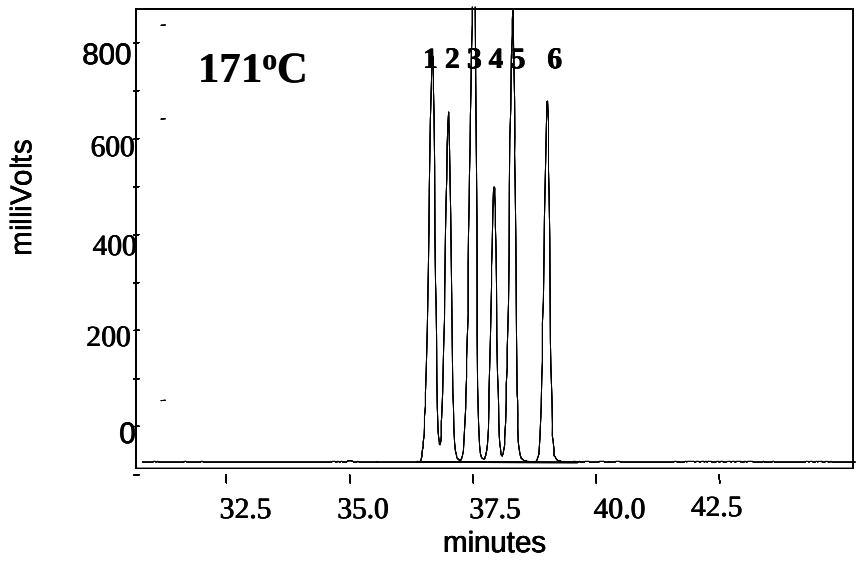



**Figure 20.2 Part of the chromatogram showing the separation of six standard 18:1 isomers analysed at a column temperature of 171°C on a 120 m BPX-70 capillary column**

**Peak identification: 1= t6, 2= t9, 3= t11, 4=c6, 5=c9, 6=c11**

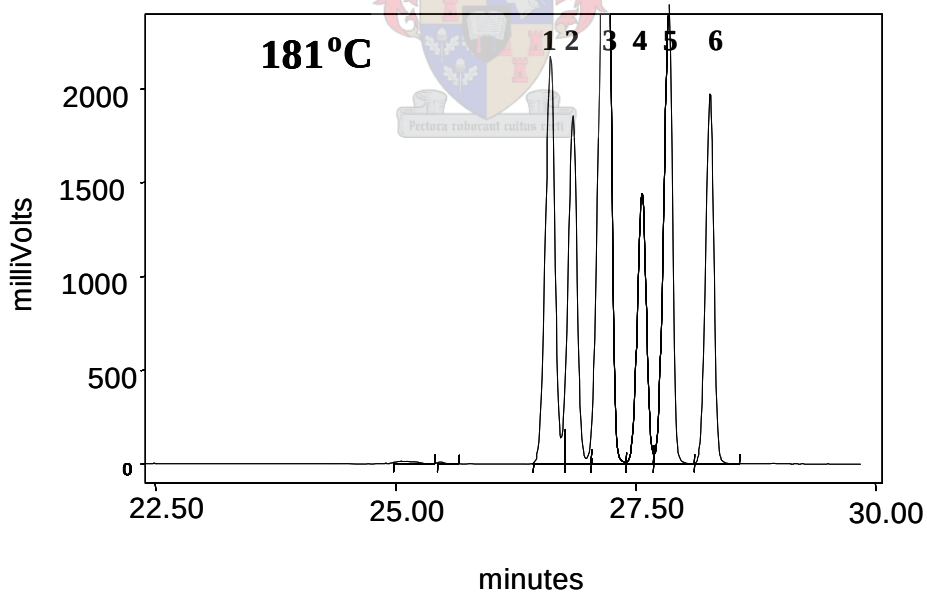



**Figure 20.3. Part of the chromatogram showing the separation of six standard 18:1 isomers analysed at a column temperature of 181°C on a 120 m BPX-70 capillary column**

**Peak identification: 1= t6, 2= t9, 3= t11, 4=c6, 5=c9, 6=c11**

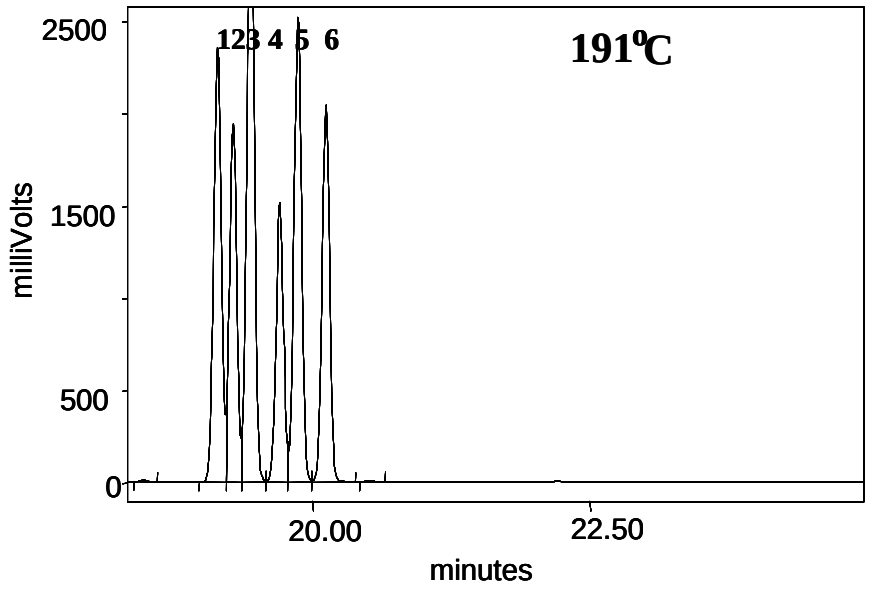



**Figure 20.4. Part of the chromatogram showing the separation of six standard 18:1 isomers analysed at a column temperature of 191°C on a 120 m BPX-70 capillary column**

**Peak identification: 1= t6, 2= t9, 3= t11, 4=c6, 5=c9, 6=c1**

The chromatogram in Figure 20.1 shows that all six isomers could be identified with baseline separation at a column temperature of 151°C. The difference in elution time (retention time) between the first isomer and the last one was 6.21 minutes. This was enough time for all the isomers to elute without overlapping, but the analyses took nearly 85 minutes. Using a column temperature of 191°C, (Figure 20.4) the analysis time was shortened to just over 20 minutes and the time difference between the first and last peak decreased to 1 minute only. Yet all the fatty acids could still be identified, though the three *trans* isomers and the *cis*-6 and *cis*-9 isomers started overlapping. With a column temperature of 181°C, (Figure 20.3) all the isomers were baseline separated with very little overlapping and the analysis time was approximately 28 minutes.

**Table 7. The effect of column temperature on the percentage composition of the different fatty acid isomers analysed with a 120 m BPX-70 capillary column**

| Isomers / Temp. | *Trans*-6 | *Trans*-9 | *Trans*-11 | *Cis*-6 | *Cis*-9 | *Cis*-11 | Total |
|---|---|---|---|---|---|---|---|
| **151°C** | **17.5** | **14.4** | **22.9** | **11.4** | **18.7** | **15.1** | **100** |
| **171°C** | **17.6** | **14.3** | **23.0** | **11.3** | **18.7** | **15.1** | **100** |
| **181°C** | **17.6** | **14.3** | **23.0** | **11.3** | **18.7** | **15.1** | **100** |
| **191°C** | **17.9** | **14.0** | **23.1** | **10.8** | **19.1** | **15.1** | **100** |
| **Average** | **17.7** | **14.3** | **23.0** | **11.2** | **18.8** | **15.1** | |
| **STD.** | **0.2** | **0.2** | **0.1** | **0.3** | **0.2** | **0.0** | |

Table 7 shows that the percentage composition of the six isomers in the standard mixture differs very little when using different column temperatures. It was only at a column temperature of 191°C that the area percentages of the different isomers differed slightly from the others. This could only be because of the overlapping of some of the peaks at the higher column temperature. These results also show that the elution order of a standard mixture of *cis* and *trans* FAME isomers, with different positional and geometrical structures do not change with different column temperatures between 151°C and 191°C and hydrogen gas flow rates between 26 and 38 cm $sec^{-1}$ using a 120 m BPX-70 capillary column. However, it shows that higher column temperatures cause some peak overlapping. From Table 7 it is evident that the concentration of the individual isomers stays constant at the three lower column temperatures, as indicated by the relatively small standard deviation in their percentage composition. It is concluded that a standard mixture of six different *cis* and *trans* FAME isomers can be separated on a 120 m BPX-70 capillary column using any column temperature between 151°C and 181°C.

Previous literature (Kramer *et al.,* 2004; Ratnayake *et al.,* 2002; Aro *et al.,* 1998; de Koning *et al.,* 2001) stated that column temperatures have a major effect on the separation of FAMEs prepared from partially hydrogenated plant oils. Not only does the column temperature have an effect on the retention times of the different fatty acid isomers, but also do some isomers

overlap when using a specific temperature. Changing the column temperature, however, causes other isomers to overlap.

To optimise the column temperature for the analyses of margarine FAMEs, the prepared pooled margarine sample was injected at different column temperatures.

## 4.5 Evaluation of different column temperatures

The nature and velocity of the carrier gas are primary considerations for the efficiency of a given column. Hydrogen was used as the carrier gas, because of its high diffusivities and low resistance to mass transfer (Christie, 1989). Different column gas flow rates between 26 cm $sec^{-1}$ and 38 cm $sec^{-1}$ were also utilised. It was found that except for a change in the retention times, there was very little effect on resolution, column efficiency and elution order of the different isomers, when hydrogen was utilised. Thus, the precise flow rate was less critical. Other authors also observed no loss of resolution, with different flow rates (Ratnayake *et al.,* 2006). This effect was also illustrated by a so-called Van Deemter plot of the variation in the height of an effective theoretical plate with carrier gas velocities for hydrogen, helium and nitrogen, where it could be seen that with hydrogen the height of the effective theoretical plate varied little, with changes in the flow rate (Figure 12 on page 32).

The GLC results of the pooled margarine FAME sample, using column temperatures between 151$^{o}$C and 197$^{o}$C, are demonstrated in the next twelve chromatograms (Figures 21.1-21.12). With a column temperature of 151$^{o}$C, (Figure 21.1) five peaks, 1-5 were separated before the main *cis*-9, 18:1 fatty acid and six peaks, 6-11 after that. With column temperatures between 155$^{o}$C and 170$^{o}$C, only four peaks could be separated before the *cis*-9, 18:1 fatty acid peak, because peak 1 and 2 overlapped. At a column temperature of 170$^{o}$C, a small peak (peak 12) became separated from peak 10. With an increase in column temperature this peak slowly moved towards peak 9 until it became part of peak 9 at a column temperature of 181$^{o}$C. With column temperatures between 175$^{o}$C and 183$^{o}$C, five peaks were again separated before the main isomer as peak 13 became separated from the *cis*-9, 18:1 fatty acid peak. A new small peak (peak 14) also became separated from peak 11, to give 8 identifiable peaks after *cis*-9, 18:1 peak. Raising the column temperature above 183$^{o}$C, peaks 3 and 4 started to overlap and peak 6 started to overlap with the main *cis*-9, 18:1 peak. At a column temperature of 197$^{o}$C,

peaks 1 and 2 and peaks 3 and 4 overlapped and the main peak overlapped peak 6, leaving only four identifiable peaks before the main isomer and six separated peaks thereafter.

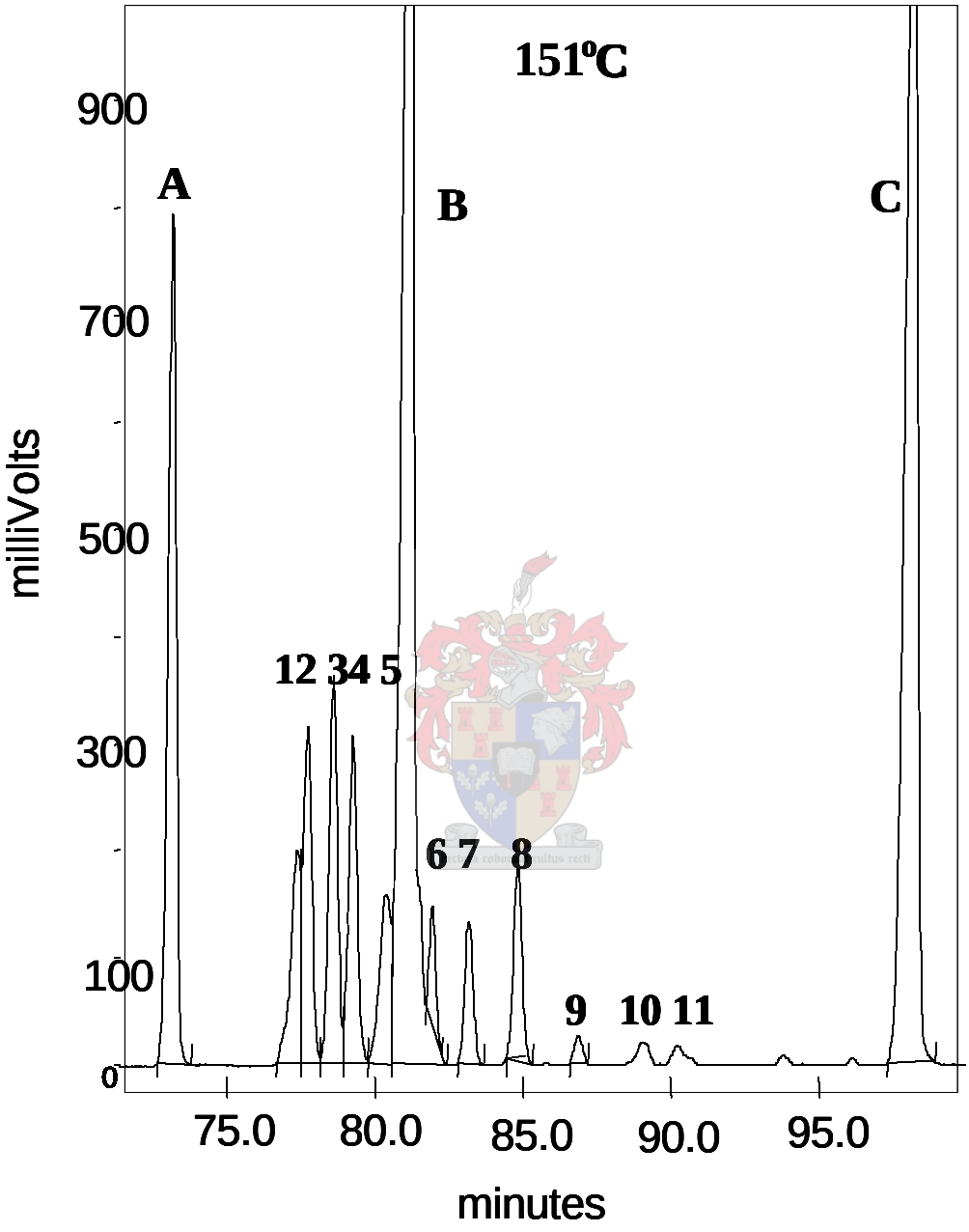
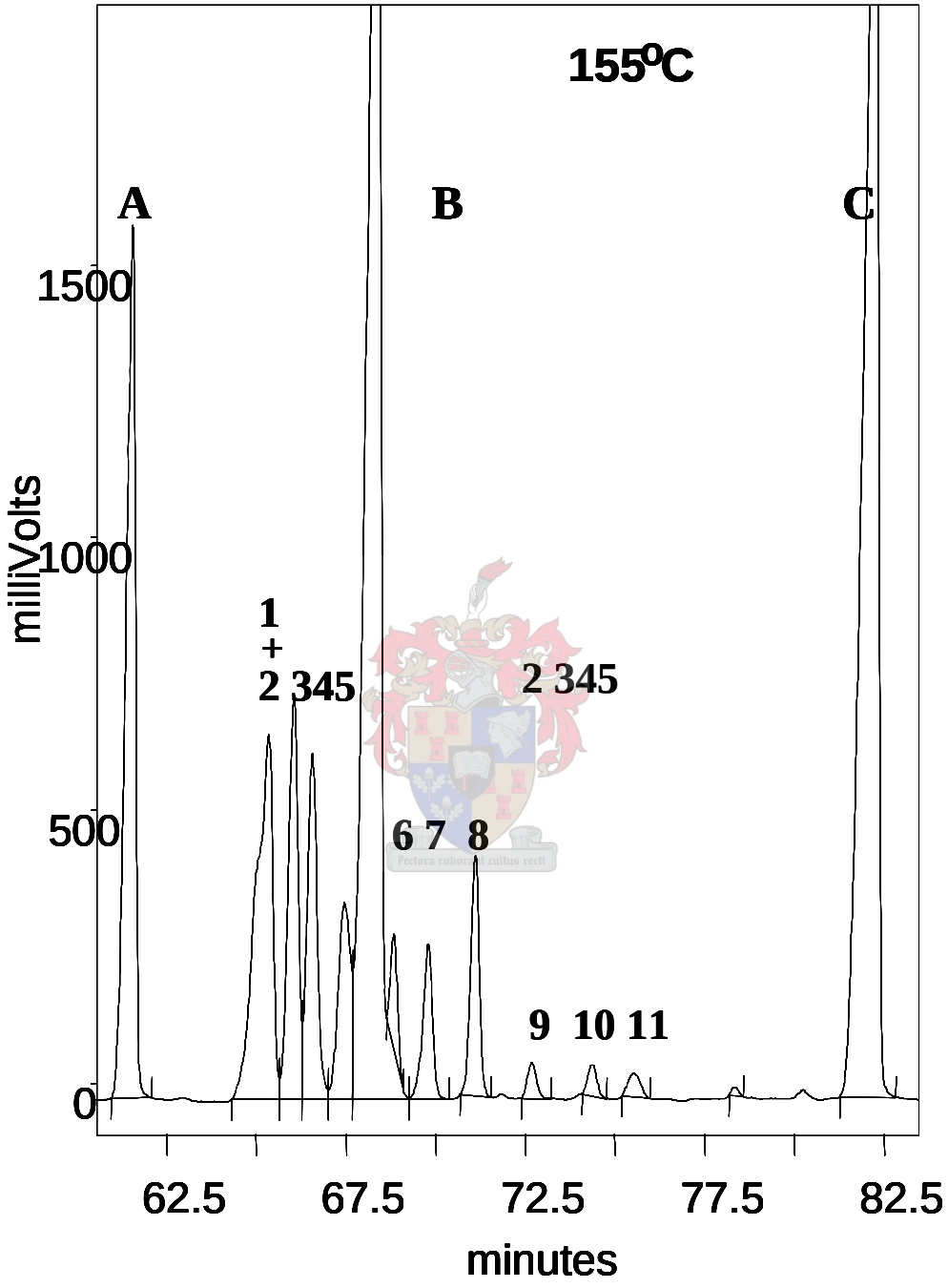



**Figure 21.1. Chromatogram of sample analysed at column temperature of 151° C (11 peaks can be separated)**




**\Figure 21.2. Chromatogram of sample analysed at column temperature of 155°C (10 peaks can be separated)**

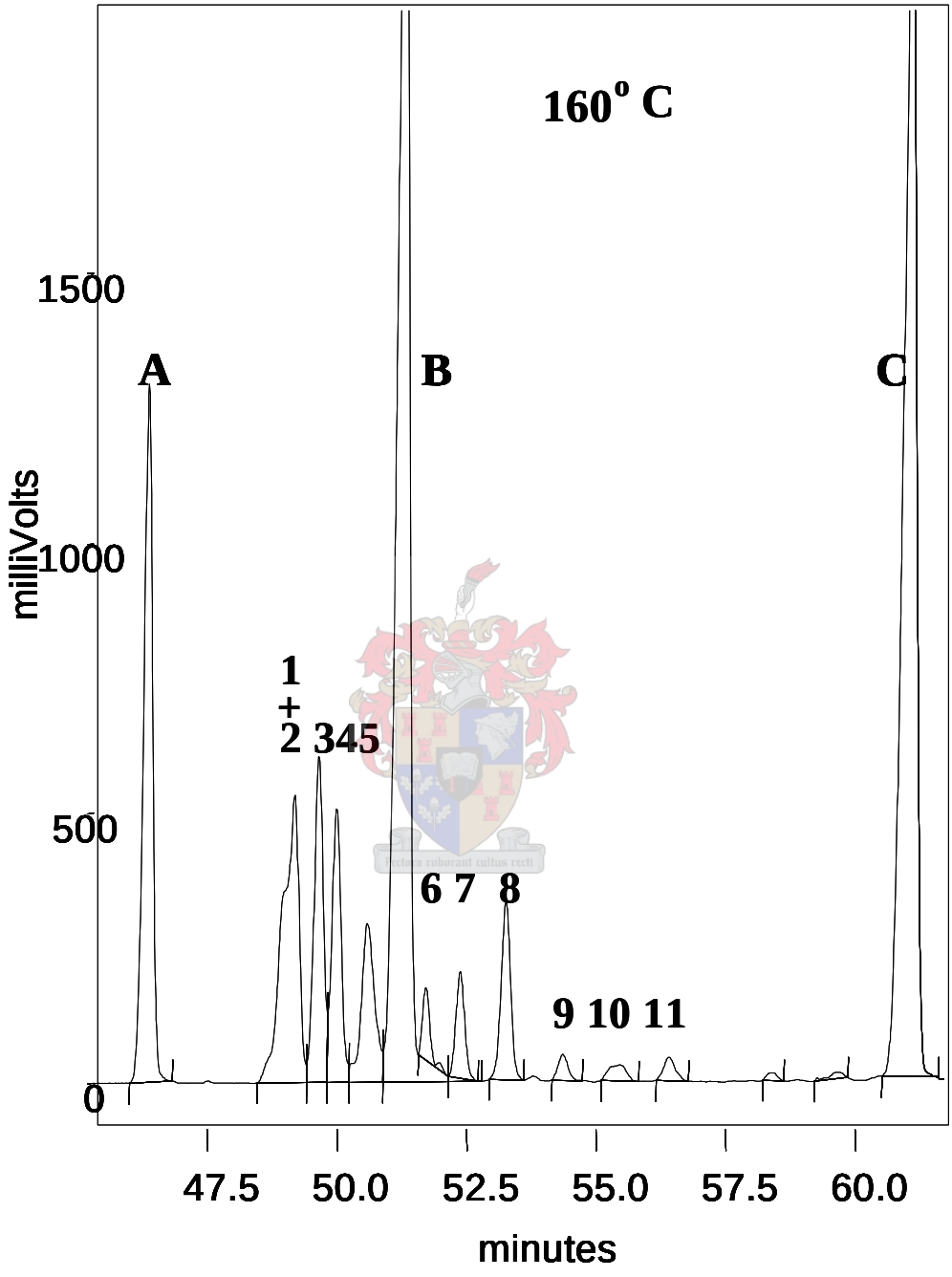



**Figure 21.3. Chromatogram of sample analysed at column temperature of 160°C (10 peaks can be separated)**

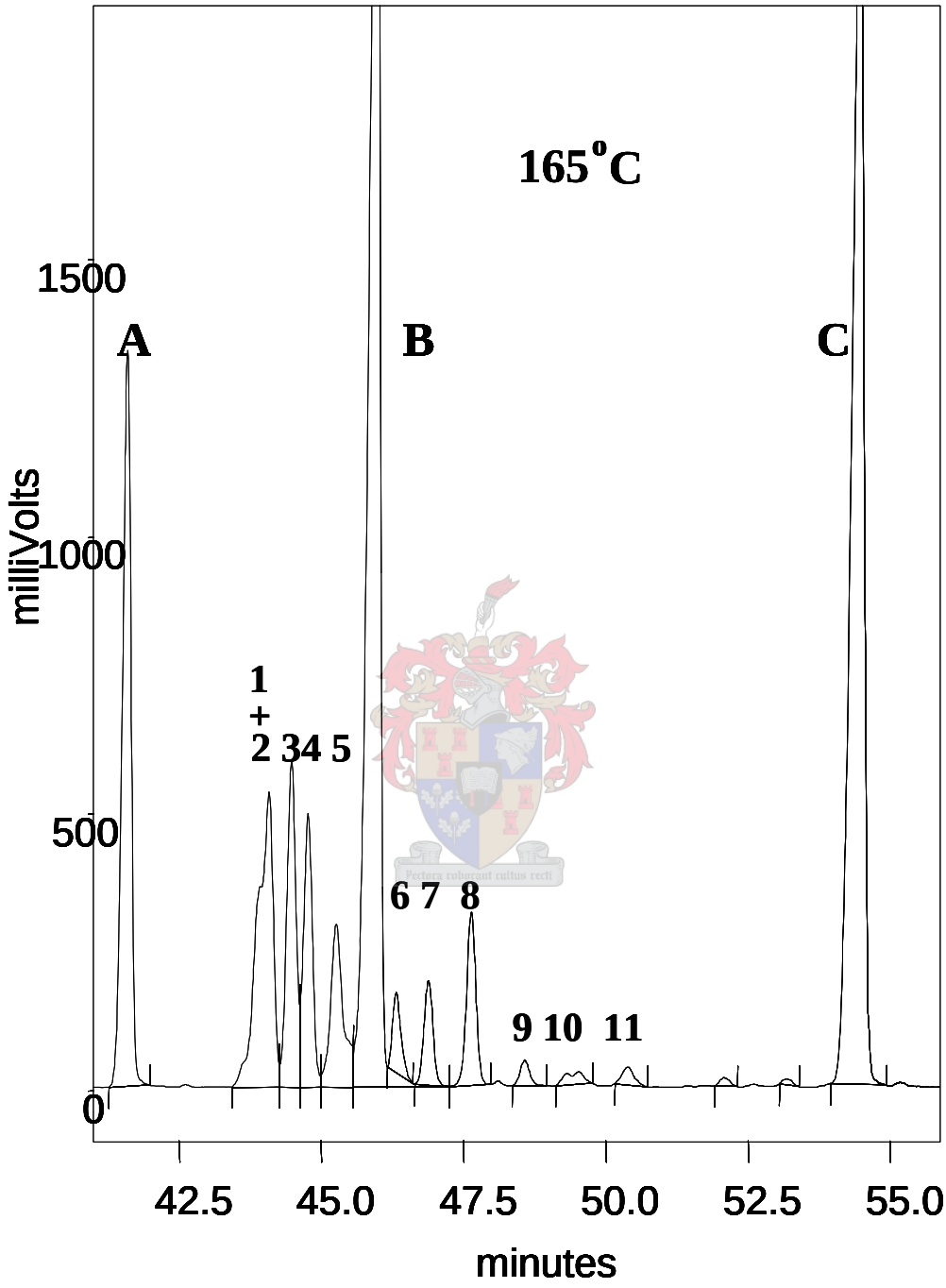



**Figure 21.4. Chromatogram of sample analysed at column temperature of 165°C (10 peaks can be separated)**

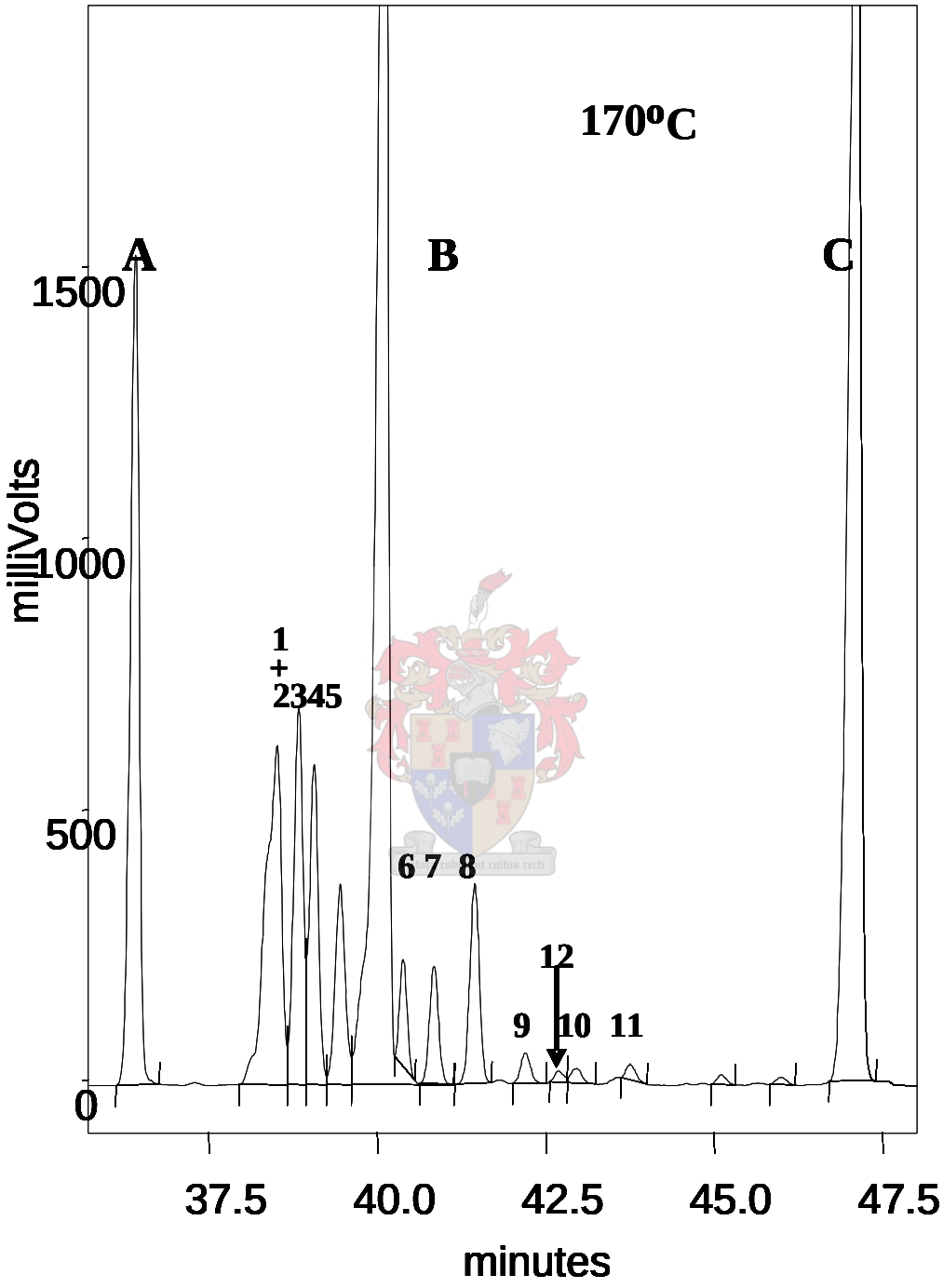



**Figure 21.5. Chromatogram of sample analysed at column temperature of 170°C (11 peaks can be separated)**

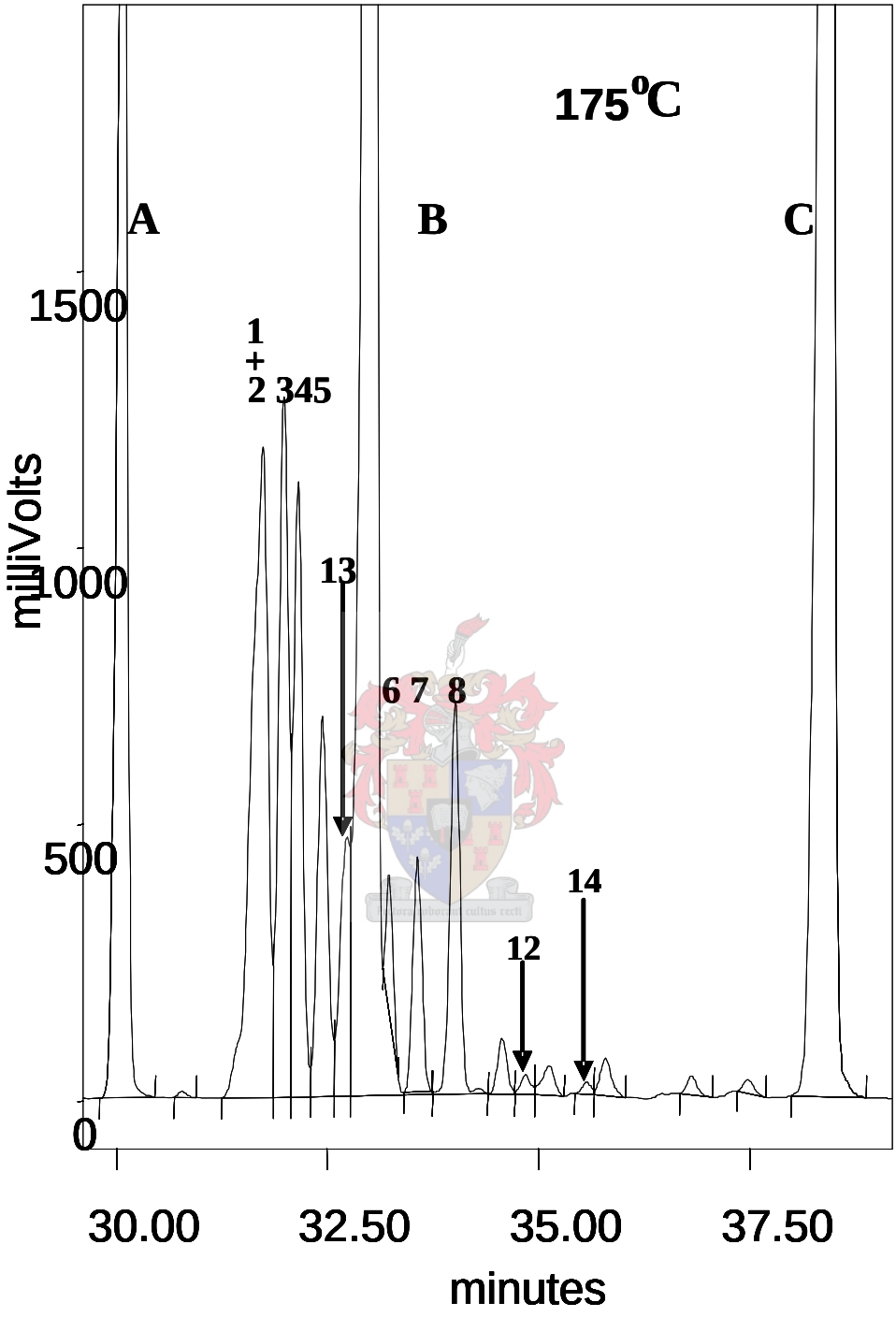



**Figure 21.6. Chromatogram of sample analysed at column temperature of 175°C (13 peaks can be separated)**

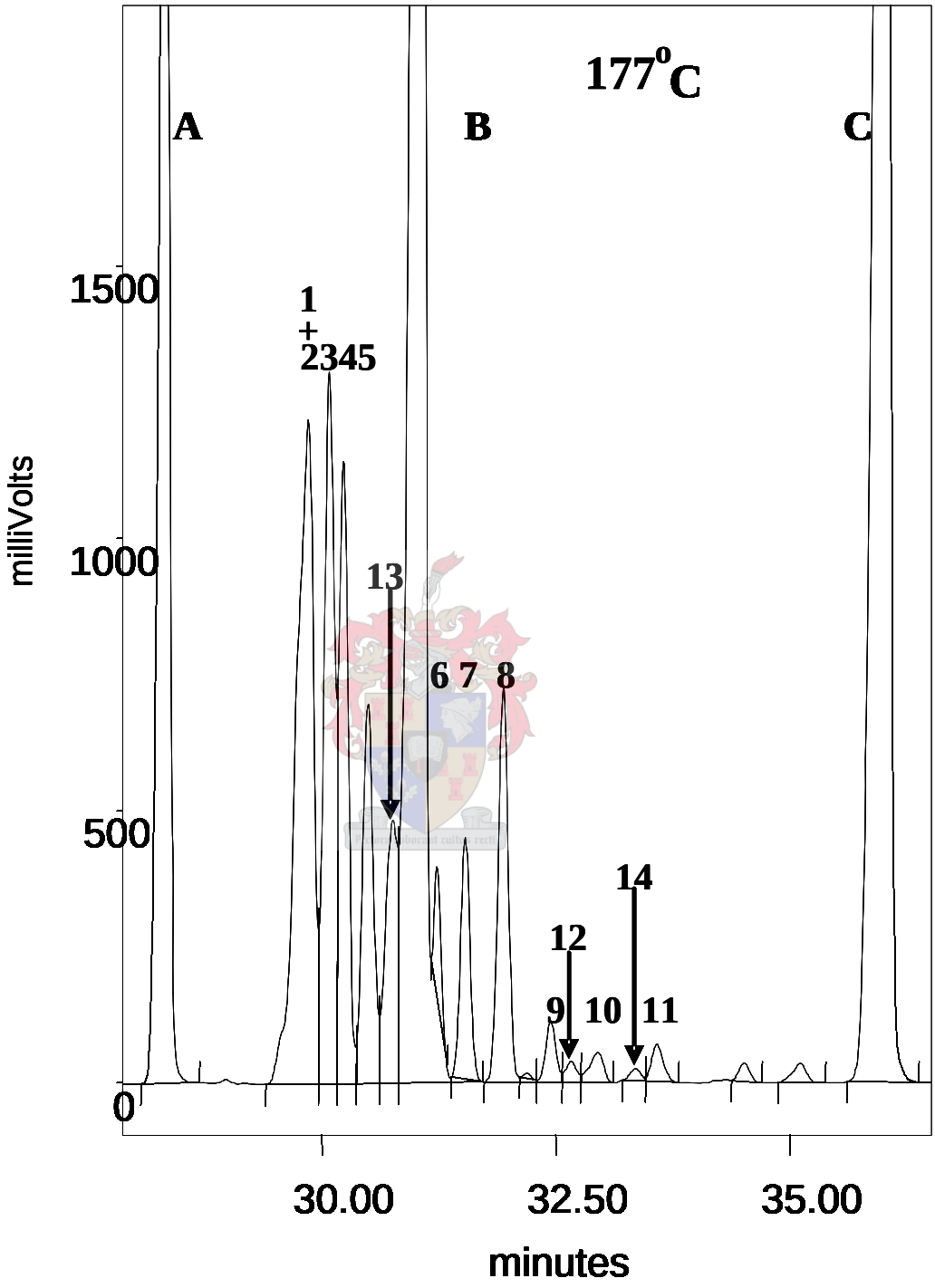



**Figure 21.7. Chromatogram of sample analysed at column temperature of 177°C (13 peaks can be separated)**

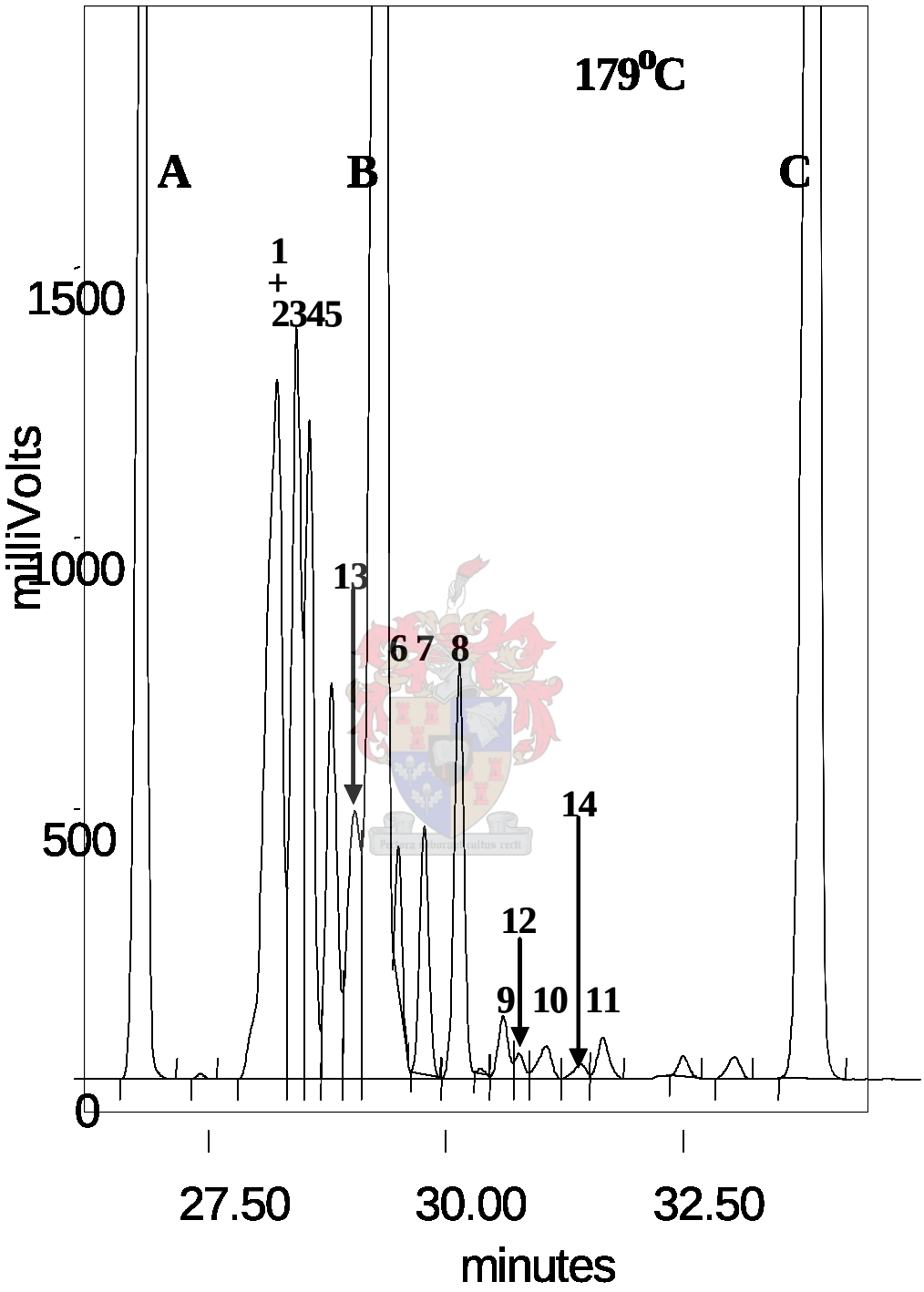



**Figure 21.8. Chromatogram of sample analysed at column temperature of 179°C (13 peaks can be separated)**

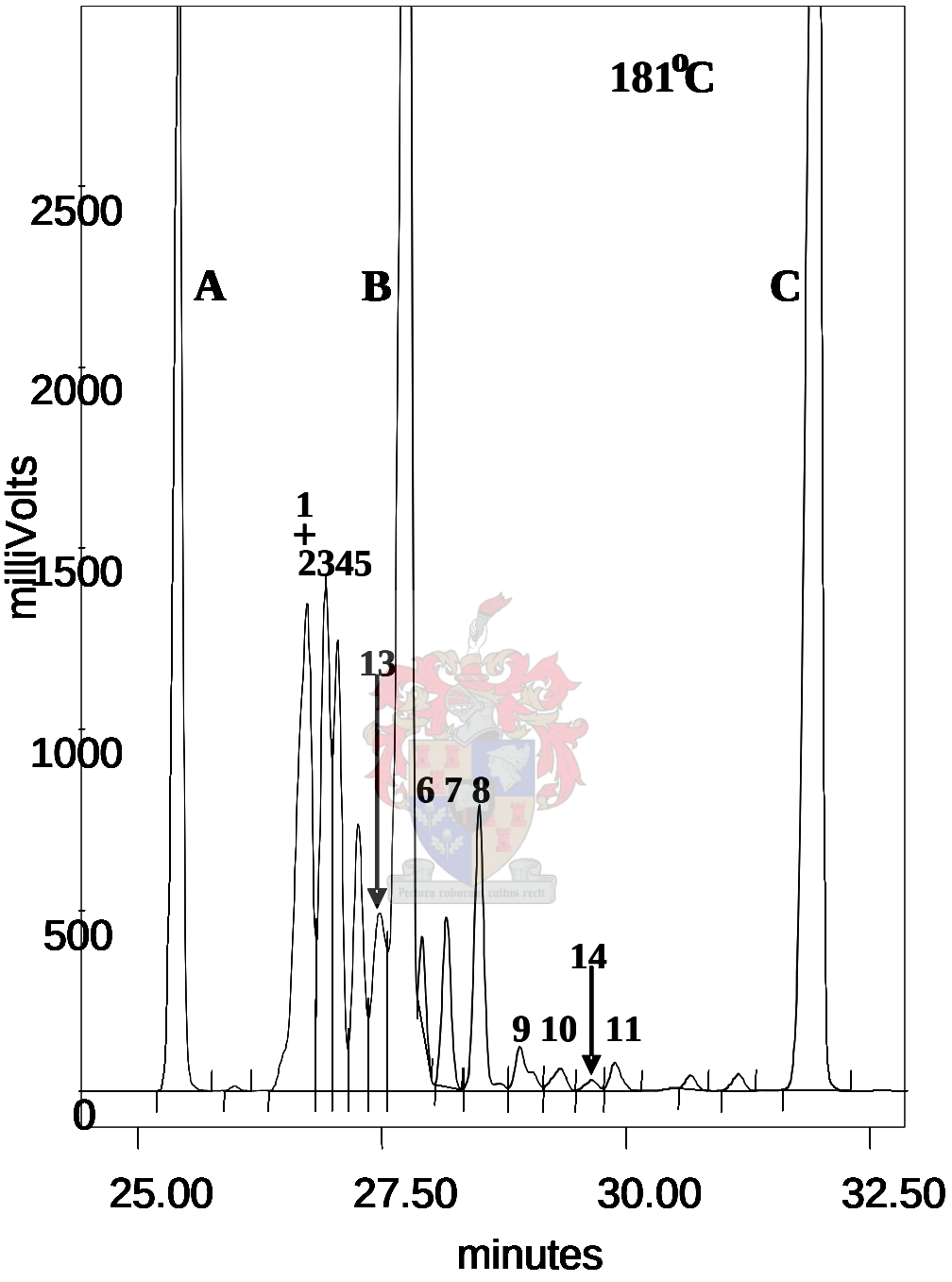



**Figure 21.9. Chromatogram of sample analysed at column temperature of 181°C (12 peaks can be separated)**

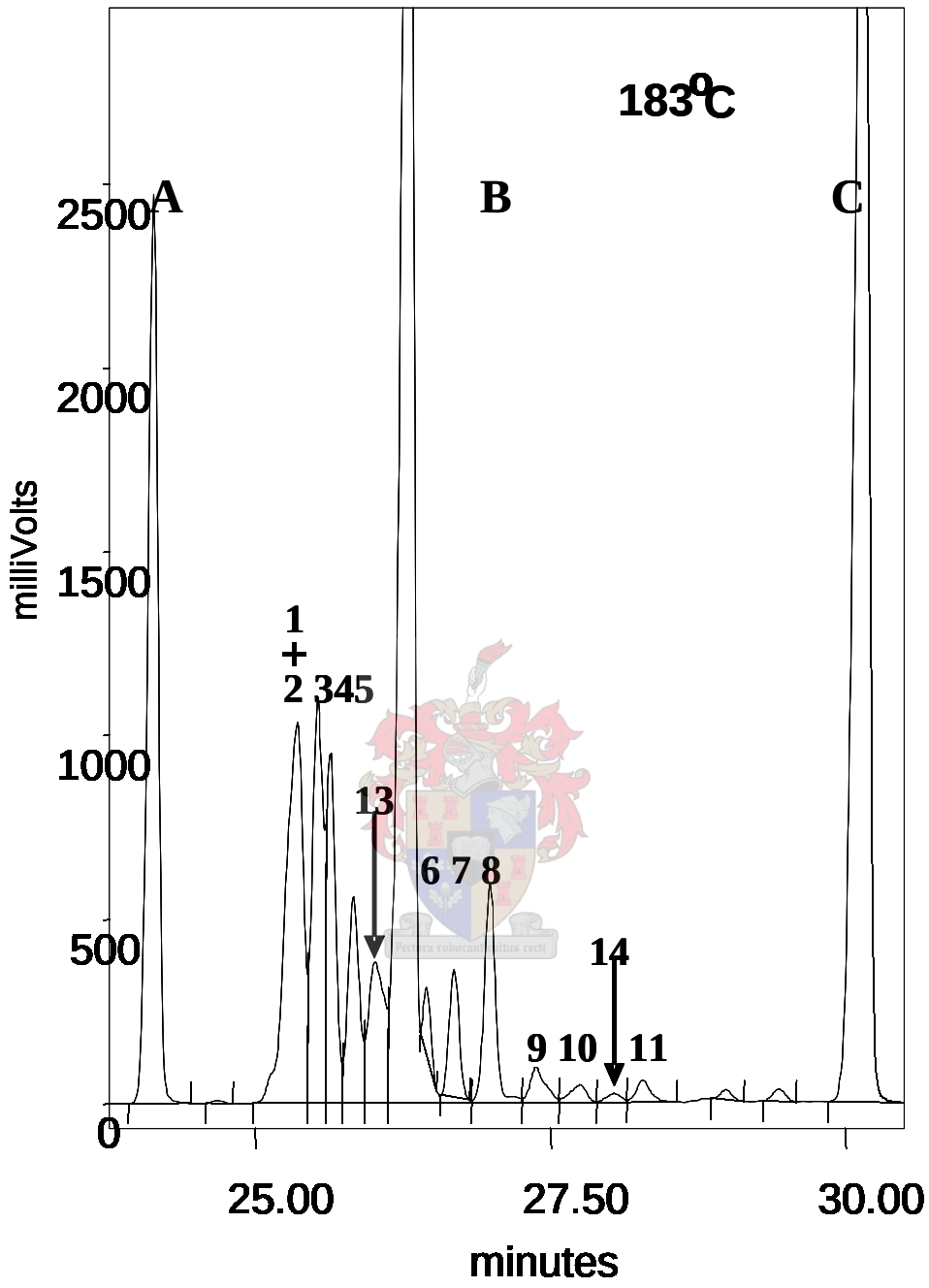



**Figure 21.10. Chromatogram of sample analysed at column temperature of 183°C (12 peaks can be separated)**

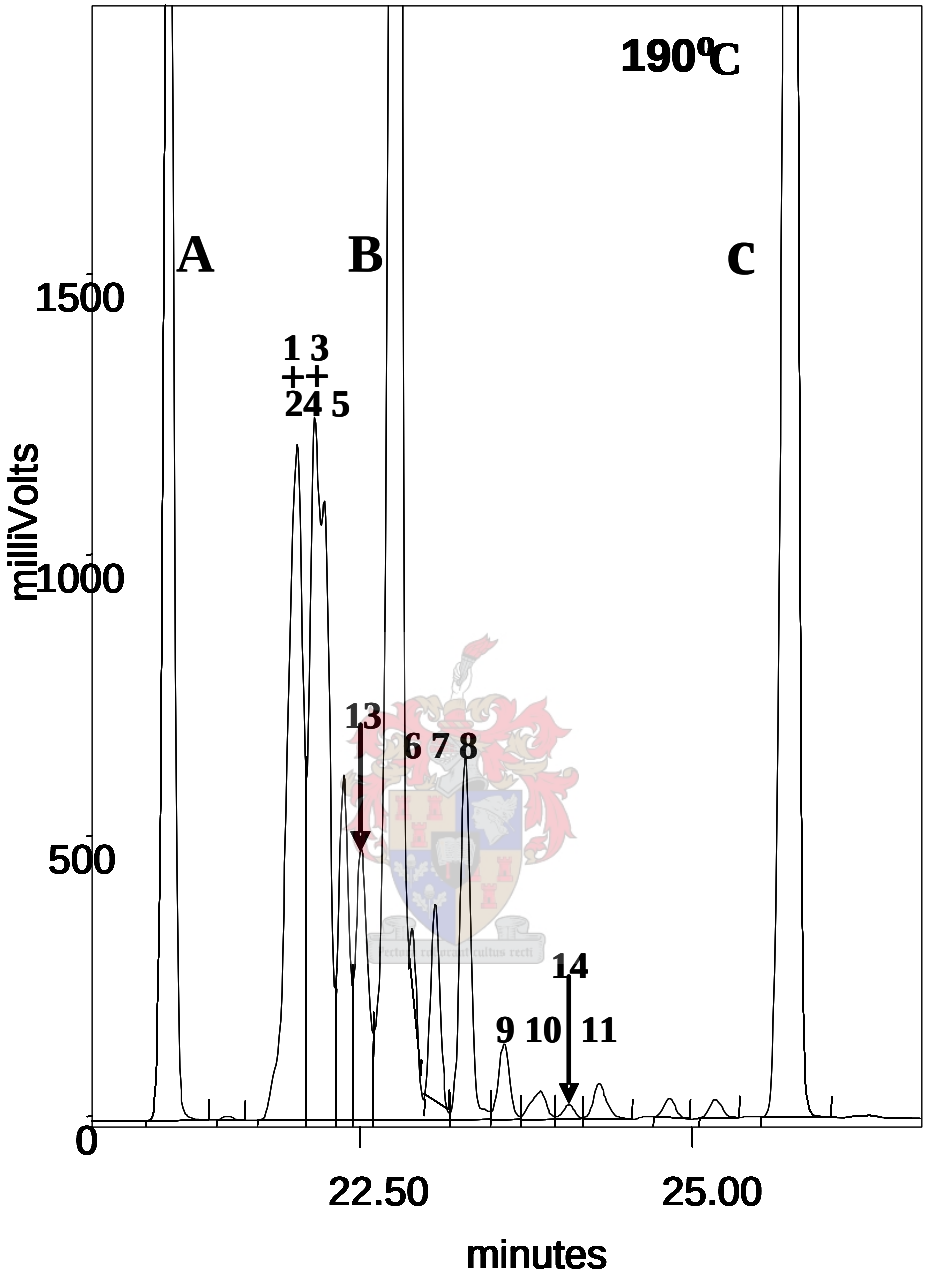



**Figure 21.11. Chromatogram of sample analysed at column temperature of 190°C (11 peaks can be separated)**

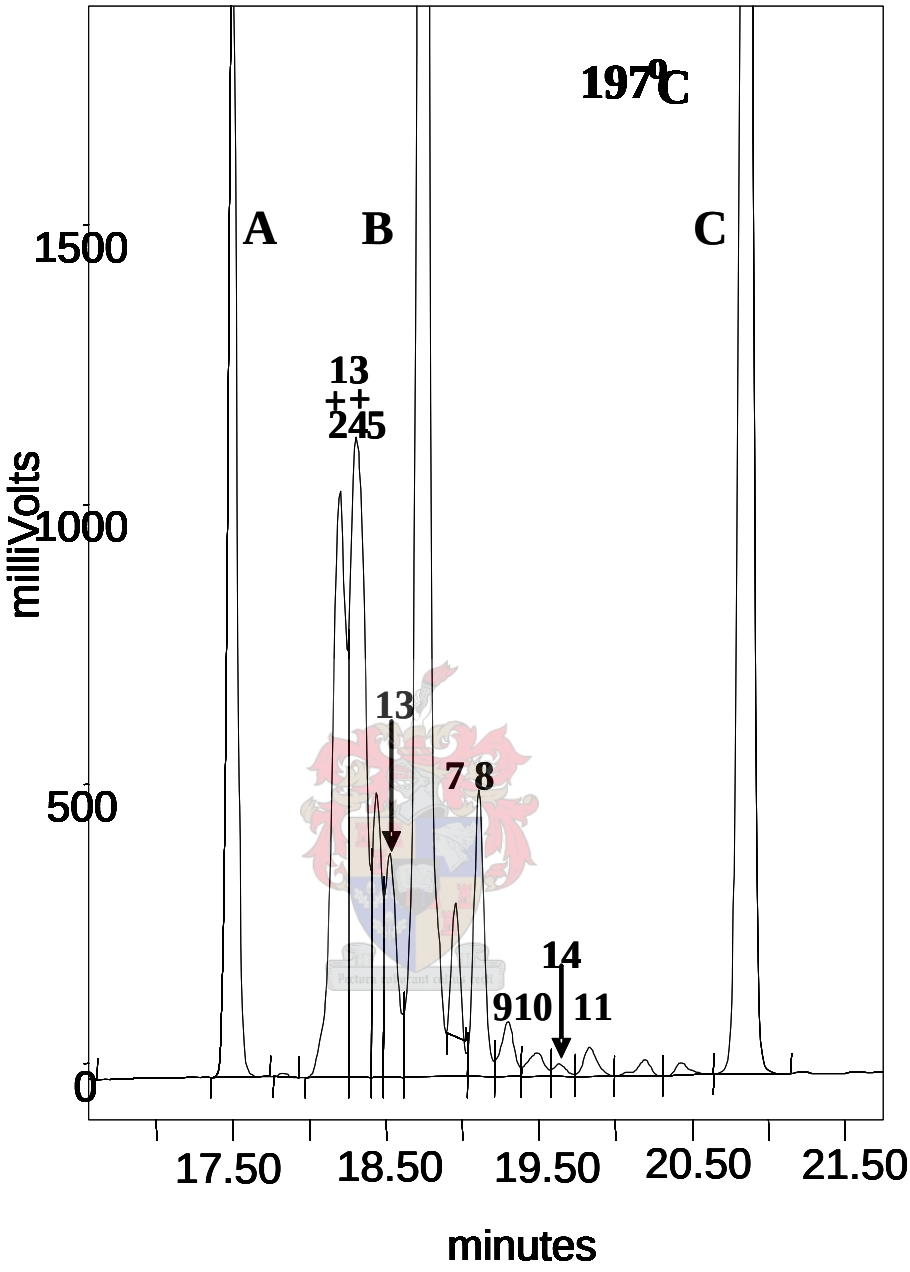



**Figure 21.12. Chromatogram of sample analysed at column temperature of 197°C (10 peaks can be separated)**

A summary of the peaks separated at the different column temperatures, as well as the temperature effect on peak areas, because of overlapping, are demonstrated in Table 5.

**Table 8. The percentage composition of the different peaks and of the main 18:1 fatty acid isomer in the pooled margarine sample, using different column temperature between 151°C and 197°C**

| Peak | 1 | 2 | 1+2 | 3 | 4 | 3+4 | 5 | 13 | 18:1 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 14 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 151°C | 2.9 | 4.1 | - | 4.6 | 4.0 | - | 2.6 | - | 25.4 | 1.1 | 1.7 | 2.4 | 0.3 | 0.3 | 0.3 | - | - |
| 155°C | - | - | 7.1 | 4.6 | 4.0 | - | 2.8 | - | 25.1 | 0.9 | 1.7 | 2.4 | 0.4 | 0.3 | 0.3 | - | - |
| 160°C | - | - | 7.0 | 4.6 | 3.7 | - | 3.2 | - | 25.2 | 0.8 | 1.4 | 2.5 | 0.3 | 0.4 | 0.4 | - | - |
| 165°C | - | - | 7.1 | 4.6 | 3.7 | - | 3.1 | - | 25.1 | 1.0 | 1.4 | 2.5 | 0.4 | 0.3 | 0.3 | - | - |
| 170°C | - | - | 7.2 | 4.7 | 3.8 | - | 2.0 | - | 25.3 | 1.1 | 1.5 | 2.5 | 0.4 | 0.2 | 0.2 | 0.1 | - |
| 175°C | - | - | 7.1 | 4.7 | 3.7 | - | 2.6 | 1.7 | 24.4 | 0.6 | 1.4 | 2.5 | 0.3 | 0.1 | 0.2 | 0.1 | 0.1 |
| 177°C | - | - | 7.1 | 4.7 | 3.6 | - | 2.6 | 1.9 | 24.2 | 0.5 | 1.4 | 2.5 | 0.4 | 0.1 | 0.3 | 0.1 | 0.1 |
| 179°C | - | - | 7.1 | 4.6 | 3.6 | - | 2.5 | 2.1 | 24.1 | 0.6 | 1.4 | 2.6 | 0.4 | 0.1 | 0.3 | 0.1 | 0.2 |
| 181°C | - | - | 7.0 | 4.7 | 3.6 | - | 2.5 | 2.2 | 24.0 | 0.5 | 1.4 | 2.6 | 0.5 | 0.3 | 0.1 | - | 0.2 |
| 183°C | - | - | 7.1 | 4.8 | 3.5 | - | 2.5 | 2.3 | 23.9 | 0.5 | 1.4 | 2.6 | 0.5 | 0.3 | 0.1 | - | 0.3 |
| 190°C | - | - | 7.0 | - | - | 8.5 | 2.3 | 2.3 | 24.6 | 0.2 | 1.2 | 2.6 | 0.5 | 0.3 | 0.1 | - | 0.3 |
| 197°C | - | - | 7.1 | - | - | 8.5 | 2.3 | 2.4 | 25.1 | - | 0.9 | 2.6 | 0.6 | 0.3 | 0.1 | - | 0.3 |

The differences in the percentage areas of some of the peaks, at different temperatures, could only be because of overlapping, since there were no significant differences in the percentage areas of the principal 16:0, 18:0 and 18:2 fatty acids peaks. Although, depending on the column temperature, between 10 and 13 different peaks could be separated. This is still less than the reported number of isomers that could be found in partially hydrogenated plant oils. The literature states that the octadecenoic acid (18:1) isomer group, which is the primary isomeric fatty acid group in partially hydrogenated plant oils, can constitute up to 26 different *cis* and *trans* isomers (Ratnayake *et al.*, 2002). Kramer *et al.* identified 12 different *trans* fatty acid isomers in a partially hydrogenated oil sample, although the peak areas of some of these peaks were very small (Kramer *et al.*, 2004). Aro *et al.* identified 13 isomers using a 100-m CP-Sil capillary column with a column temperature of 170°C. Preceding Ag-TLC, improved their separation to 16 isomers (Aro *et al.*, 1998).

It was concluded that even with a highly-polar 120 m BPX-70 column, it was not possible to separate all the different isomers in the margarine samples using only one isothermal column temperature. Using temperature programming will not solve this problem, because the retention times of the different *cis* and *trans* isomers are very close to one another, leaving very little time for temperature programming. It has been reported that temperature programming gives less satisfactory separation of *cis*- and *trans*- 18:1 isomers, whereas optimal isothermal column conditions provide far more improved separation of the 18:1 isomers, although some isomers will always overlap (Ratnayake *et al.*, 2002). Ideally, each sample will have to be injected at different column temperatures a number of times. This will be very time consuming and still not guarantee the separation and identification of all the *cis* and *trans* isomers in the samples.

The chromatograms (Figures 21.1-21.12) and the evaluation of the percentage area counts of the different peaks (Table 8), demonstrated that between 175$^{o}$C and 183$^{o}$C the most individual peaks could be identified, although not all the peaks were baseline separated. At a column temperature of 175$^{o}$C, peak 13 became separated from the main isomer and the separation improved with a raise in column temperature. On the other hand, at 175$^{o}$C, peak 6 was still separated, though with a rise in temperature it started to overlap the main *cis*-9, 18:1 isomer. Decreasing the column temperature to 151$^{o}$C, resulted in a better separation between the early eluting *trans* isomers, but the later eluting isomers were not well resolved and the analytical time was very long. The average sum of all the isomers, between the different column temperatures, was 49.6 ± 0.3%. Therefore, choosing a column temperature, which gives the highest total percentage of all the different isomers in a sample, will be of little use, because the average total concentration of the isomers between the different column temperatures was nearly the same. Therefore, it was decided to use a column temperature of 181$^{o}$C for the identification and quantification of the different isomers in the margarine samples, since the main *cis*-9, 18:1 fatty acid overlapped the fewest isomers at this temperature, and the analytical time was reasonable.

The identification of the individual fatty acid methyl ester peaks was done by comparing the retention times of the sample peaks with those obtained from *cis* and *trans* FAME standards (Nu- Chek- Prep, INC. Elysian, Minnesota, USA). These were analysed under exactly the same analytical conditions as used for the sample. To help with the identification of

overlapping peaks, the sample was spiked with known FAME standard isomers. To further assist with the identification, equivalent chain-length (ECL) values for *cis* and *trans* FAMEs from SGE were used (www.sge.com). The quantification of the different fatty acids was done with the 17:0 internal standard added to the sample.

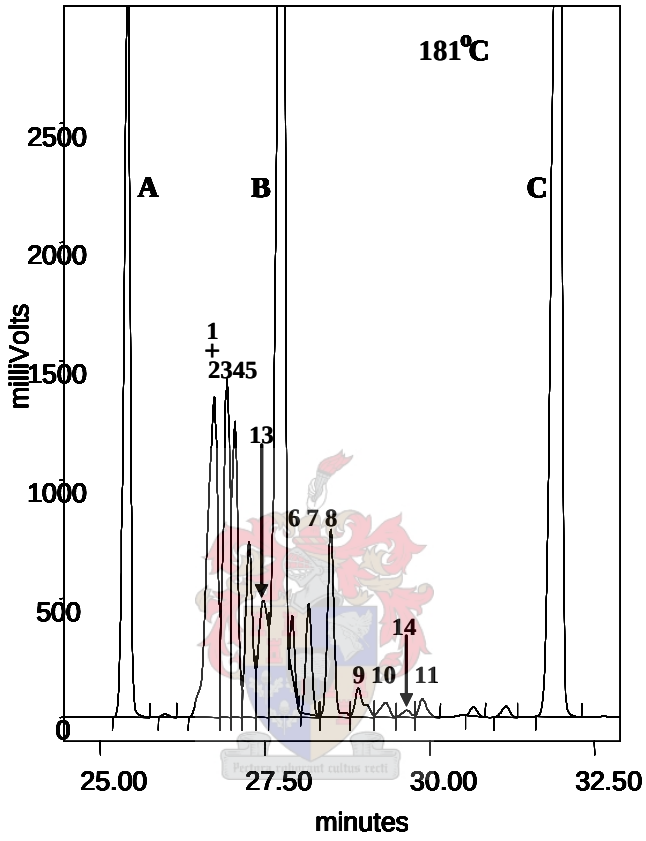



**Figure 21.13. Chromatogram of the pooled sample analysed at column temperature of 181°C**

In the 181°C chromatogram (Figure 21.13) the first peak was identified by spiking to be at least the unresolved *trans*-6 and *trans*-9 isomers. This overlapping was confirmed in the chromatogram of the analyses at a column temperature of 151°C, (Figure 21.1) where it was confirmed to be two peaks representing at least two isomers. *Trans*-7 and -8 could not be identified, but the literature states that the *trans*-6, -7 and -8 isomers are always eluting as one peak (Precht *et al.*, 1996; Ratnayake *et al.*, 2002), thus peak 1 was identified as the unresolved *trans*-6, -7, -8 and -9 isomers. Peaks 3, 4 and 5 were identified as *trans*-10, -11 and -12. Peak 13 was identified as the unresolved *trans*-13 and -14 isomers, because using a highly-polar,

very long capillary column, the *trans*-13 and -14 isomers always elute as one peak (Precht *et al.*, 1996; Ratnayake *et al.*, 2002). The peak marked B is the main *cis*-9, 18:1 isomer. The *cis*-6, -7 and -8 isomers that normally elute before the *cis*-9, could not be separated. After spiking the sample with a *cis*-6 FAME standard, it was found that the *cis*-6 isomer forms part of the unresolved *trans*-13 and -14 isomers' peak. This observation was confirmed by Ratnayake *et al.*, who found that the unresolved *trans*-13 and -14 isomers' peak always overlaps the *cis*-6, -7 and -8 isomers when using a long highly-polar column. These 3 *cis* isomers are of minor importance in the analyses of partially hydrogenated plant oil samples, because they contained less than 0.2% of these three *cis* isomers (Ratnayake *et al.*, 2002). The *trans*-15 isomer could not be identified, and it was calculated that its retention time was very close to the retention time of the *cis*-9, 18:1 isomer, and that this large peak is probably overlapping it. Quantitatively, this is not a major drawback, because the *trans*-15 isomer is a minor component in partially hydrogenated plant oils (Glew *et al.*, 2006; Ratnayake *et al.*, 2002). Peaks 6-11 were identified as *cis*-10, -11, -12, -13, -14 and -15, respectively. Another noteworthy improvement with a 120 m BPX-70 column was the partial separation of *trans*-16 (peak 14) and *cis*-15 (peak 11). The *trans*-16 isomer could only be resolved at an isothermal column temperature above 175$^{o}$C. Normally this isomer appeared as a shoulder on the edge of the *cis*-15 isomer peak (Ratnayake *et al*., 2002). Except for the *cis*-16 isomer, all the other peaks were identified.



Table 9 gives the results of the identification and quantification of the different peaks analysed at 151$^{o}$C, 170$^{o}$C, 181$^{o}$C and 197$^{o}$C. Although, some of the *trans* isomers eluted as a group as well as some overlapping between *cis* and *trans* isomers, elaidic acid (*trans*-9, 18:1) which is the major man-made *trans* fatty acid found in partially hydrogenated plant oils and processed foods (Belury, 2002) and vaccenic acid (*trans*-11, 18:1) that occurs naturally in foods from animal sources (Wolff, 1995), could be identified. The two saturated fatty acids, 16:0 and 18:0, and the one polyunsaturated fatty acid, 18:2, in the sample were well resolved with column temperatures between 151$^{o}$C and 197$^{o}$C. The average concentrations of these fatty acids between the different column temperatures were 16:0; 14.7 ± 0.2 mg/100 mg, 18:0; 8.3 ± 0.1 mg/100 mg and the 18:2; 19.6 ± 0.2 mg/100 mg. The concentration range of the main *cis*-9, 18:1 fatty acid isomer, between the different column temperatures was between 20.4 and 21.5 mg/100 mg. This large difference was because of the overlapping with peak 13 at a lower temperature, and peak 6 at a temperature of 197$^{o}$C. The total fatty acid

concentration of the margarine, as determined at a column temperature of 181$^{o}$C, was 85.2 mg/100 mg. This fatty acid concentration is the highest of the four column temperatures that were evaluated, indicating that selecting a column temperature of 181$^{o}$C for the analyses of the samples is correct. The total *trans* fatty acid concentration, at a column temperature of 181$^{o}$C, was 17.3%. The *cis*-6, -7, and -8 isomers that possibly overlapped with the *trans*-13 and -14 were calculated as *trans* isomers. The *trans*-15 isomer, which the *cis*-9 isomer overlapped, was calculated as *cis*-9, 18:1.

Table10 gives the results of the pooled sample that was injected five times into a 120 m BPX-70 capillary column with a column temperature of 181$^{o}$C. The total fatty acid concentration of the sample was 84.8 ± 0.2 mg/100 mg and the average concentration of the major *trans* isomers (*trans*-6 to *trans*-14) was 17.2 ± 0.2 mg/100 mg. The low standard deviation of the individual saturated and polyunsaturated fatty acids, as well as the different *cis* and *trans* 18:1 isomers indicate good reproducibility. The percentage coefficient of variance was also less than 2% for the major *cis* and *trans* isomers, except for those with low concentrations where this statistical measurement is not a good tool.

**Table 9. The total fatty acid concentration in mg/100 g of the pooled margarine sample injected into a 120 m BPX-70 capillary column at different column temperatures between 151°C and 197°C**

| | 16:0 | 18:0 | 18:1 | | | | | | | | | | | | | | | | 18:2 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | t6,t7 t8 | t9 | t6,t7 t8,t9 | t10 | t11 | t10,t11 | t12 | t13,t14 c6,c7,c8 | c9 t15 | c9,c10 t15 | c10 | c11 | c12 | c13 | c14 | c15 | t16 | | |
| 151°C | 15.0 | 8.4 | 2.4 | 3.5 | - | 3.9 | 3.4 | - | 2.2 | - | 21.5 | - | 0.9 | 1.4 | 2.0 | 0.3 | 0.3 | 0.3 | - | 19.6 | 85.1 |
| 170°C | 14.7 | 8.3 | - | - | 6.0 | 3.9 | 3.2 | - | 1.6 | - | 21.1 | - | 0.9 | 1.2 | 2.1 | 0.3 | 0.2 | 0.3 | - | 19.6 | 83.5 |
| 181°C | 14.8 | 8.3 | - | - | 6.0 | 4.0 | 3.1 | - | 2.1 | 1.9 | 20.4 | - | 0.4 | 1.2 | 2.2 | 0.5 | 0.2 | 0.2 | 0.2 | 19.8 | 85.2 |
| 197°C | 14.5 | 8.3 | - | - | 6.0 | - | - | 7.1 | 1.9 | 1.9 | - | 21.1 | - | 0.8 | 2.2 | 0.5 | 0.3 | 0.2 | 0.3 | 19.7 | 84.9 |
| AVG | 14.8 | 8.3 | - | - | 6.0 | 3.9 | 3.2 | - | 2.0 | 1.9 | 21.0 | - | 0.7 | 1.1 | 2.1 | 0.4 | 0.3 | 0.3 | - | 19.7 | 84.7 |
| STD | 0.2 | 0.0 | - | - | 0.0 | 0.1 | 0.2 | - | 0.2 | 0.0 | 0.6 | - | 0.3 | 0.3 | 0.1 | 0.1 | 0.1 | 0.1 | - | 0.1 | 0.8 |

**Table 10. The total fatty acid concentration in mg/100 mg of the pooled margarine sample injected five times into a 120 m BPX-70 capillary column at a column temperature of 181$^{o}$C**

| | 16:0 | 18:0 | 18:1 | | | | | | | | | | | | | 18:2 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | t6,t7 t8,t9 | t10 | t11 | t12 | t13,t14 c6,c7,c8 | c9 t15 | c10 | c11 | c12 | c13 | c14 | c15 | t16 | | |
| A | 14.7 | 8.3 | 6.0 | 3.9 | 3.1 | 2.2 | 1.7 | 20.4 | 0.5 | 1.2 | 2.1 | 0.3 | 0.2 | 0.2 | 0.2 | 19.8 | 84.9 |
| B | 14.8 | 8.3 | 6.0 | 4.0 | 3.1 | 2.2 | 1.6 | 20.8 | 0.5 | 1.2 | 2.2 | 0.3 | 0.2 | 0.2 | 0.3 | 19.8 | 84.9 |
| C | 14.7 | 8.3 | 6.1 | 3.9 | 3.1 | 2.2 | 1.8 | 20.7 | 0.5 | 1.2 | 2.2 | 0.3 | 0.2 | 0.3 | 0.2 | 19.8 | 84.9 |
| D | 14.8 | 8.3 | 6.0 | 4.0 | 3.1 | 2.1 | 1.9 | 20.6 | 0.4 | 1.2 | 2.2 | 0.5 | 0.2 | 0.3 | 0.2 | 19.8 | 85.1 |
| E | 14.5 | 8.2 | 6.1 | 4.0 | 3.0 | 2.2 | 2.0 | 20.6 | 0.4 | 1.1 | 2.2 | 0.5 | 0.2 | 0.3 | 0.2 | 19.5 | 84.4 |
| AVG | 14.7 | 8.3 | 6.0 | 4.0 | 3.1 | 2.2 | 1.8 | 20.6 | 0.4 | 1.2 | 2.2 | 0.4 | 0.2 | 0.3 | 0.2 | 19.7 | 84.8 |
| STD | 0.1 | 0.1 | 0.0 | 0.0 | 0.1 | 0.0 | 0.2 | 0.1 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.2 | 0.2 |
| %CV | 0.8 | 0.7 | 0.8 | 1.0 | 1.9 | 1.8 | 8.4 | 0.7 | 7.8 | 2.7 | 1.1 | 21.1 | 6.1 | 5.0 | 20.3 | 0.8 | 0.3 |

## 4.6 Results of silver ion thin layer chromatography

Figure 22.1 shows a photograph of the developed Ag-TLC plate. The *trans* (1) and *cis* (2) mono-unsaturated fatty acid fractions were well separated with no overlapping between the two fractions. The fractions were analysed using the same GLC conditions that was used to identify and quantify the unfractioned pooled sample.

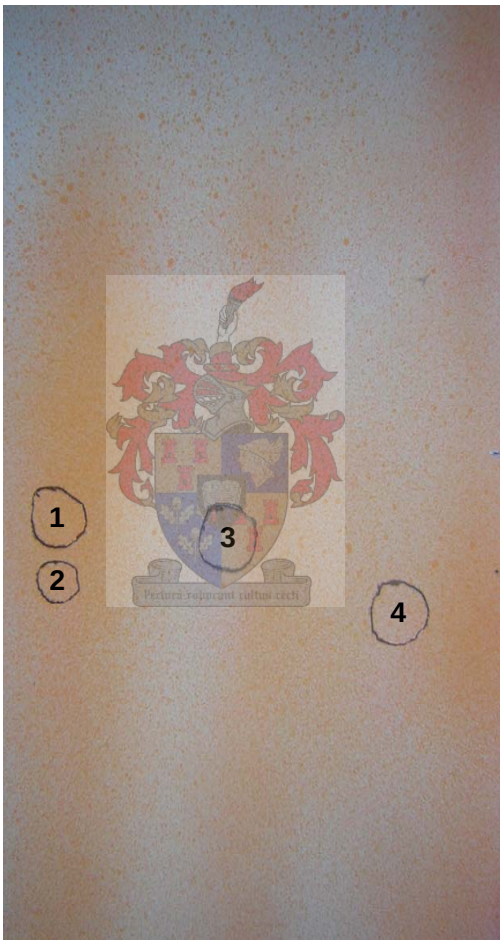



**Figure 22.1. Photograph of a TLC plate impregnated with 10% (w/v) silver nitrate showing the separation of the *cis* and *trans* monounsaturated FAME isomer fractions in the pooled margarine sample**

**1= Trans isomers. 2= Cis isomers. 3= *Trans*-9, 18:1 standard. 4= *Cis*-9, 18:1 standard**

Part of the GLC chromatogram of the *trans* mono- unsaturated fatty acid fraction is given in Figure 22.2.

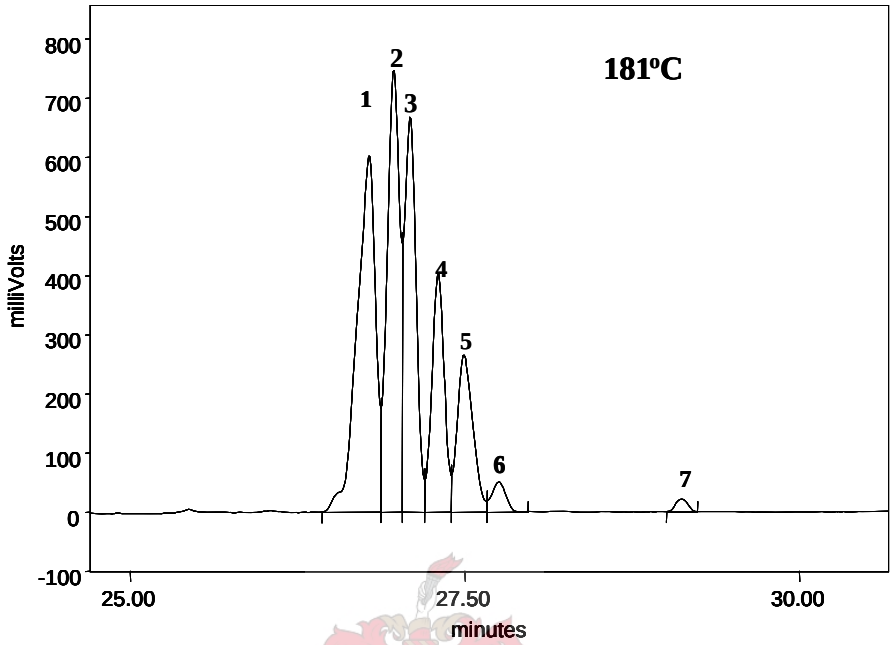



**Figure 22.2. Part of the chromatogram of the *trans* mono-unsaturated FA fraction of the pooled sample, after Ag-TLC separation. The fraction was analysed with a 120 m BPX-70 capillary column at a column temperature of 181°C**

**Peak identification: 1= *trans*- 6, 7, 8 and 9, 2=*trans* -10, 3= *trans*- 11, 4= *trans*- 12, 5= *trans*-13 and 14, 6= *trans*-15 and 7=*trans*-16**

Seven different peaks could be identified from the chromatogram of the *trans* fraction (Figure 22.2). The *trans*-6, -7, -8 and -9 isomers still eluted as one peak (peak 1). Peaks 2-4 were identified as *trans*-10, -11 and -12. Peak 5 is the unresolved *trans*-13 and -14 isomers. Peak 6 is the *trans*-15 isomer that is normally overlapped in the unfractioned samples by the main *cis*-9 isomer, and peak 7 is the *trans*-16 isomer. None of the known resolved *cis* isomers were present in this fraction, proving that Ag-TLC separated the *cis* and *trans* mono-unsaturated fatty acid isomers completely, but the different overlapping *trans* isomers were still not separated. This overlapping effect of the *trans* isomers was also reported by other authors (Precht *et al*., 1996; Aro *et al*., 1998).

The GLC results (in percentage compositions) of the different *trans* isomers, with and without fractionation with Ag-TLC, are summarised in Table. 11.

**Table 11. The GLC results (in percentage composition) of the different *trans* 18:1 isomers, with and without preceding Ag-TLC separation.**

| Isomers / Method | *Trans* 6 - 9 | *Trans* 10 | *Trans* 11 | *Trans* 12 | *Trans* 13+14 | *Trans* 15 | *Trans* 16 | Total (%) |
|---|---|---|---|---|---|---|---|---|
| Without Ag-TLC | 33.7 | 23.9 | 19.4 | 13.2 | 10.4 | -- | 0.4 | 100 |
| With Ag-TLC | 32.0 | 24.0 | 19.2 | 13.3 | 8.8 | 2.2 | 0.5 | 100 |

The same *trans* overlapping groups and individual isomers, with the exception of *trans*-15, were identified. The difference of 1.7% between the "without Ag-TLC" and the "with Ag-TLC" groups of the *trans*-6 to *trans*-9 isomeric group is likely because of the overlapping effect of the *cis*-5 isomer that was identified in the *cis* mono-unsaturated fatty acid fraction after Ag-TLC. The other noteworthy difference between the two methods was the well-resolved *trans*-15 isomer, after Ag-TLC. With its 2.2 % composition of the total *trans* fatty acids it could be seen that this *trans* isomer is not as negligible as some authors have reported (Ratnayake *et al*., 2002). There is very little difference in the percentage compositions of the *trans*-10, -11 and -12 isomers between the two methods. This is an indication that there are no *cis* isomers eluting with the *trans*-10, -11 and -12 isomers in the unfractioned sample.

The chromatogram (Figure 22.3) of the *cis* mono-unsaturated fatty acid fraction showed 11 different peaks.

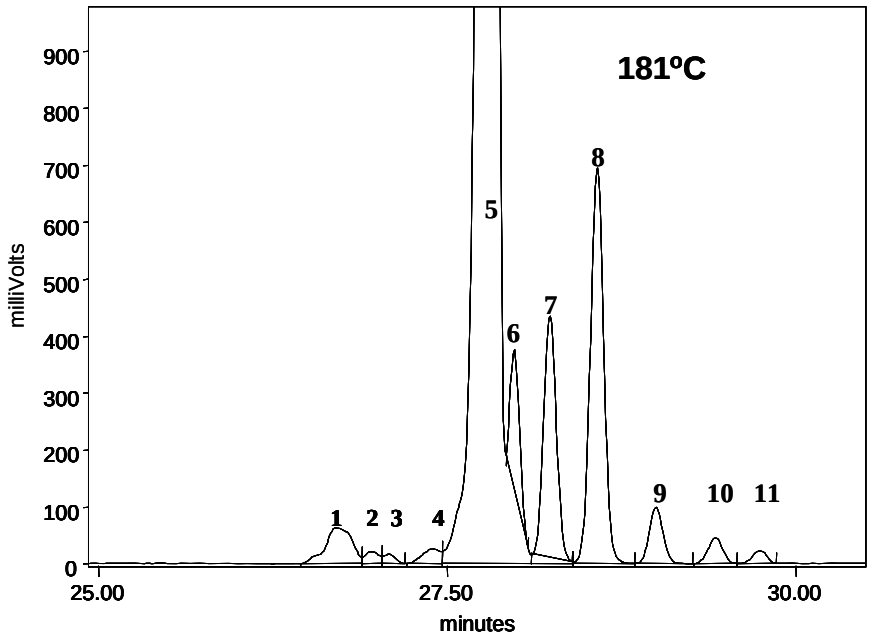



**Figure 22.3. Part of the chromatogram of the *cis* mono-unsaturated fraction of the pooled sample, after Ag-TLC separation. The fraction was analysed with a 120 m BPX-70 capillary column at a column temperature of 181°C**

**Peak identification: 1= cis-5, 2=*cis*-6, 3=*cis*-7, 4=*cis*-8, 5= *cis*-9, 6= *cis*-10, 7=*cis*-11, 8=*cis*-12, 9=*cis*-13, 10=*cis*-14 and 11=*cis*-15**

The well-resolved peak 1 has not been mentioned in the most recent and prominent publications on *trans* fatty acids in partially hydrogenated vegetable oils (Precht *et al*., 1996; Ratnayake *et al*., 2002; Glew *et al*., 2006; Kramer *et al*., 2004; de Koning *et al*., 2001; Ratnayake *et al*., 2006; Ledoux *et al*., 2000). From this peak's retention time, it must elute together with the overlapping group of *trans*-6 to *trans*-9 isomers in the unfractioned samples. In the fractioned sample, this isomer elutes just before the known group of *cis*-6 to *cis*-8 isomers and, therefore, the assumption can be made that it is probably a *cis*-5 isomer. However, it could not be proven, because a *cis*-5 standard was not available to confirm this. To exclude the possibility of a contaminant, the extraction and separation was repeated with similar results. Peaks 2, 3 and 4 were identified as the *cis*-6, -7 and -8 isomers that are normally overlapped by the *trans*-13 and -14 isomers. The separation of these three *cis* isomers, although not very good, was noted. In the *trans* mono-unsaturated fatty acid fraction, it was not possible to separate any of the known overlapping *trans* groups. Peaks 5, 6, 7, 8, 9, 10 and 11 were identified as the *cis*-9 to *cis*-15 isomers.

**Table 12. The GLC results (in percentage composition) of the different *cis* 18:1 isomers, with and without preceding Ag-TLC separation**

| Isomers / Method | *Cis* 5 | *Cis* 6 | *Cis* 7 | *Cis* 8 | *Cis* 9 | *Cis* 10 | *Cis* 11 | *Cis* 12 | *Cis* 13 | *Cis* 14 | *Cis* 15 | Total (%) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Without Ag-TLC** | -- | -- | -- | -- | **81.4** | **1.6** | **4.8** | **8.9** | **1.6** | **0.7** | **1.0** | **100** |
| **With Ag-TLC** | **1.6** | **0.3** | **0.2** | **0.4** | **78.6** | **2.2** | **5.2** | **9.1** | **1.4** | **0.5** | **0.5** | **100** |

Table 12 gives the percentage composition of the *cis* isomers determined by the two methods. With Ag-TLC separation, all the *cis* isomers that eluted before the main cis-9 isomer could be identified, but were overlapped by the *trans* isomers in the unfractioned samples. The difference in the percentage composition between the *cis*-9 isomer in the two methods was caused by the overlapping effect of the *trans*-15 isomer in the unfractioned sample. With Ag-TLC separation, the *trans*-15 did not form part of the *cis*-9 isomer and, therefore, its percentage composition was 2.8% lower. With the exception of the *cis*-5 to *cis*-8 isomers, no other new isomers were separated. The 0.5% difference in the composition of the *cis*-15 between the two methods is an indication that there was some overlapping with the *trans*-16 isomer in the unfractioned sample. Except for these few discrepancies, the percentage composition of the other isomers between the two methods was very comparable.

The different isomers in the two fractions were not quantified, because Ag-TLC was only used to separate the *cis* and *trans* isomeric groups and to see to what extent overlapping of these isomers can influence the concentration of the different isomers in the unfractioned samples. To quantify the different isomers, two internal standards with known concentrations, a *cis* mono-unsaturated and a *trans* mono-unsaturated fatty acids, which are not occurring in the samples, must be used. Unfortunately, with partial hydrogenation any number of different isomers can be formed, making it impossible to predict which isomers do not occur in the sample. Another method of quantifying the different isomers is to spike the sample by pipetting precisely two aliquots of the sample extract into two transmethylation tubes. Known concentrations of a *cis*-9, 18:1 standard, and a *trans*-9, 18:1 standard, should be added to one

of the tubes. These two standard isomers are used because they are normally the main *cis* and *trans* isomers in partially hydrogenated oil samples. These duplicate samples are then analysed and from the differences in area counts of the *cis*-9, 18:1 and the *trans*-9, 18:1 peaks in the two aliquots, the concentration of the unspiked peak can be calculated. If the concentration of one peak is known, the concentration of all the other peaks can be calculated. However, Ag-TLC separations are laborious and to duplicate each sample is impractical for routine analyses.

## 4.7 Results of the margarine samples

After evaluation and standardisation of the method, the margarine samples were analysed using a 120 m BPX-70 capillary column at a column temperature of 181$^{o}$C and a hydrogen gas flow of 30 cm sec$^{-1}$.

The results (Table 13) show that only two of the samples (Sample D (18.4 mg100mg) and Sample J (2.3 mg/100 mg)) have a *trans* fatty acid concentration higher than 2% of the total fatty acids. Sample D was exceptionally high with a total *trans* content of 18.4 %. Samples A, B, C, E, G, H, I and K have also traces of *trans* fatty acids. Although the *trans* fatty acid concentration is very low it still indicates the presence of partially hydrogenated vegetable oil, because unhydrogenated vegetable oils have no *trans* fatty acids. The mean *trans* fatty acid content of the 18 margarines was 1.3 mg/100 mg fatty acids. This is much lower than the mean levels of 16.4 ± 2.6 mg/100mg reported for New Zealand (Lake *et al*, 1996) and 9.7 mg/100mg (standard deviation was not given) for the Czech Republic (Brat *et al*, 2000). Except for samples D and J that obviously contain partially hydrogenated vegetable oil, none of the other samples contained *trans*-11 isomers. This indicates that the margarines contain no animal fats, because *trans*-11, 18:1 is the main naturally occurring *trans* isomer in animal fat and milk (Sommerfeld, 1983). Samples B, C and N have high concentrations of palmitic acid (16:0) and oleic acid (18:1) and low linoleic acid (18:2) indicating that they are most probably manufactured from palm oil (Oils and Fats, 2005). The total fatty acid concentration in the 18 margarine samples varied between 44.1 and 98.3 mg/100 mg. This is directly related to the total fat content of the samples. From the fat content of the samples only three of these can be classified as margarines, because South African regulations require that margarine must at least contain 80% fat (Draft Regulations Relating to Labelling and Advertising of Foodstuffs

2002, no. R 1055). The rest of the samples, with less than 80% fat, are classified as “Table spreads”. To manufacture a “Lite” margarine (Sample M) that normally has a lower fat content, the manufacturers substitute some plant oils and fats with water. These “Lite” margarines are normally softer, indicating higher polyunsaturated fatty acid content.

**Table 13. The total fatty acid composition (mg/ 100 mg) of the margarine samples analysed with a 120 m BPX-70 capillary column at a column temperature of 181$^{o}$C and a hydrogen gas flow rate of 30 cm sec$^{-1}$**

| | 12:0 | 14:0 | 16:0 | 16:1 | 18:0 | 18:1 | | | | | | | | | | | | | 18:2 | Total |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | t6,t9 (t7,t8)* | t10 | t11 | t12 | t13,t14 (c6,c7,c8)* | c9 (t15)* | c10 | c11 | c12 | c13 | c14 | c15 | t16 | | |
| A | 2.9 | 1.4 | 15.2 | 0.1 | 2.7 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 20.7 | 0.0 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 18.9 | 62.6 |
| B | 0.2 | 0.9 | 40.2 | 0.1 | 4.0 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 32.6 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.1 | 86.8 |
| C | 0.3 | 1.2 | 49.9 | 0.1 | 5.1 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 33.1 | 0.0 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.0 | 98.3 |
| D | 0.0 | 0.2 | 16.1 | 0.0 | 8.7 | 6.3 | 4.2 | 3.2 | 2.3 | 2.1 | 21.5 | 0.5 | 1.2 | 2.3 | 0.5 | 0.3 | 0.3 | 0.3 | 20.4 | 90.4 |
| E | 2.2 | 1.2 | 18.9 | 0.1 | 2.6 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 18.5 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 11.1 | 55.0 |
| F | 2.4 | 1.4 | 24.1 | 0.1 | 2.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 21.0 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 5.3 | 57.2 |
| G | 4.9 | 2.3 | 16.9 | 0.1 | 3.8 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 16.5 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 9.4 | 54.3 |
| H | 5.1 | 2.6 | 23.1 | 0.1 | 5.4 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 23.6 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 13.7 | 74.0 |
| I | 1.0 | 1.1 | 16.7 | 0.0 | 2.4 | 0.6 | 0.4 | 0.0 | 0.1 | 0.0 | 16.5 | 0.0 | 0.3 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 5.0 | 44.1 |
| J | 2.6 | 1.8 | 25.9 | 0.1 | 3.7 | 1.3 | 0.5 | 0.3 | 0.2 | 0.0 | 26.1 | 0.0 | 0.5 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 8.4 | 71.6 |
| K | 4.7 | 3.1 | 25.2 | 0.1 | 3.8 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 23.6 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 8.5 | 69.5 |
| L | 1.7 | 1.0 | 16.9 | 0.1 | 2.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 15.3 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 7.9 | 45.3 |
| M | 1.3 | 0.9 | 7.1 | 0.0 | 6.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 14.9 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 36.0 | 66.8 |
| N | 0.1 | 0.8 | 30.8 | 0.1 | 3.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 30.6 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 9.9 | 76.3 |
| O | 2.2 | 1.7 | 21.0 | 0.1 | 4.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 21.2 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 14.8 | 65.3 |
| P | 2.1 | 1.4 | 15.0 | 0.1 | 2.7 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 20.5 | 0.0 | 0.6 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 18.7 | 61.2 |
| Q | 4.1 | 2.2 | 22.2 | 0.1 | 3.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 21.4 | 0.0 | 0.4 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 9.9 | 63.4 |
| R | 0.9 | 1.0 | 24.7 | 0.1 | 3.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 25.8 | 0.0 | 0.5 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 10.3 | 66.3 |

*** The isomers in the brackets cannot be identified because of overlapping**

The mean saturated fatty acid content of the samples (Table 14) was 45.1 mg/100 mg. According to nutritional recommendations by health authorities, the content of saturated fatty acids in margarines and table spreads should not exceed 30% in dietary fats (Brat *et al.*, 2000). However, in 16 samples the saturated fatty acids were higher. Obviously, partially hydrogenated oils were replaced by palm oil or by oils from palm seeds (high in saturated fatty acids) in some of these margarines. To increase the melting point of the margarines, without hydrogenation, the manufacturers must increase the saturated fat content and/or decrease the unsaturated fatty acid content. This is illustrated in Table 14 when comparing, for example, Sample B and C (high saturated FA, low polyunsaturated FA) with a typical soft margarine, Sample M (low saturated FA, high polyunsaturated FA). To keep to the classification of a high unsaturated fat content and a higher melting point, the manufacturers tend to keep the *cis*-9, 18:1 fatty acids as high as possible. This is well demonstrated in Samples B, C, F, J and I (high mono-unsaturated FA, low polyunsaturated FA).

**Table 14. The sum of the saturated, mono-unsaturated and polyunsaturated fatty acids (mg/ 100 mg) of the margarine samples analysed with a 120 m BPX-70 capillary column at a column temperature of 181$^{o}$C and a hydrogen gas flow rate of 30 cm sec$^{-1}$**

| Sample | Sat FA | Mono FA | Poly FA | Trans FA | Total |
|---|---|---|---|---|---|
| A | 35.6 | 34.1 | 30.2 | 0.1 | 100 |
| B | 52.2 | 38.5 | 9.3 | 0.1 | 100 |
| C | 57.4 | 34.5 | 8.1 | 0.1 | 100 |
| D | 27.6 | 31.3 | 22.6 | 18.4 | 100 |
| E | 45.2 | 34.5 | 20.3 | 0.1 | 100 |
| F | 53.2 | 37.6 | 9.2 | 0.0 | 100 |
| G | 51.6 | 31.1 | 17.3 | 0.1 | 100 |
| H | 48.8 | 32.7 | 18.5 | 0.1 | 100 |
| I | 47.9 | 39.7 | 11.4 | 1.1 | 100 |
| J | 47.6 | 38.3 | 11.8 | 2.3 | 100 |
| K | 53.0 | 34.6 | 12.3 | 0.1 | 100 |
| L | 48.2 | 34.4 | 17.4 | 0.0 | 100 |
| M | 23.3 | 22.9 | 53.8 | 0.0 | 100 |
| N | 46.0 | 41.0 | 13.0 | 0.0 | 100 |
| O | 44.1 | 33.2 | 22.7 | 0.0 | 100 |
| P | 34.7 | 34.7 | 30.6 | 0.0 | 100 |
| Q | 49.9 | 34.5 | 15.6 | 0.0 | 100 |
| R | 44.6 | 39.8 | 15.5 | 0.0 | 100 |
| AVR | 45.1 | 34.9 | 18.9 | 1.2 | |
| STD | 8.9 | 4.1 | 10.6 | 4.2 | |

Table 14 shows that Sample D contains 72.4% unsaturated fatty acids and 27.6% saturated fatty acids, giving it a much better unsaturated to saturated fatty acid ratio than for example, Sample P, which contains 7.1% less unsaturated fatty acids and 7.1% more saturated fatty acids. However, Sample D contains 18.4% *trans* unsaturated fatty acids, while Sample P contains no *trans* fatty acids, making Sample D less desirable. During routine analyses using a normal 30 m BPX-70 column, these *trans* isomers would not have been separated from the *cis*-9 18:1 fatty acid and they would have been added to the mono-unsaturated fatty acids. This would have given Sample D a high mono-unsaturated fatty acid composition of 49.8% compared to only 34.7% for Sample P, but the real *cis* mono-unsaturated fatty acid composition of Sample D is 31.3%, 1.4% less than Sample P. This illustrates that by using a short BPX-70 column, the fatty acid composition of the samples can be misinterpreted.

The part of a chromatogram in Figure 23.1 shows the fatty acids' composition of margarine Sample P. All the main fatty acid peaks are well resolved. There are no detectable peaks between 18:0 (peak A) and 18:1 (peak B), nor between 18:1 and 18:2 (peak C). The exception is a small peak under the bracket, that was identified as *cis*-11, 18:1. This isomer is not caused by partial hydrogenation, because it was also detected in blood samples that were analysed in our laboratory using a 30 m BPX-70 column. These results illustrate that margarine, Sample P, has no *trans* mono-unsaturated fatty acids.

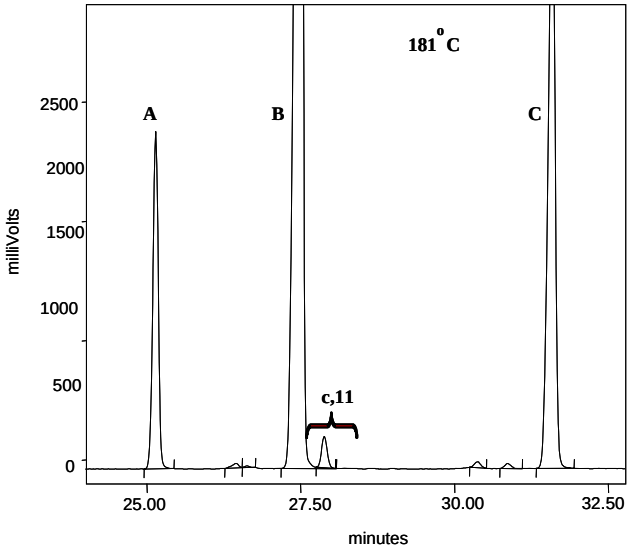



**Figure 23.1. Part of the GLC chromatogram of sample P showing the 18:0 (A), 18:1 (B) and 18:2 (C) fatty acids, as well as a *cis*-11 isomer (under the bracket)**

The next part of a chromatogram (Figure 23.2) shows the fatty acids' composition of margarine, Sample D. The normal occurring fatty acids, peak A (18:0), peak B (18:1) and peak C (18:2) are well resolved. This chromatogram clearly illustrates that this sample contains unnatural *cis* and *trans* isomers (under the bracket) indicating that this margarine was made from partially hydrogenated oil. Although not all the *cis* and *trans* isomers could be baseline separated, the main isomers formed during partial hydrogenation can be identified and quantified.

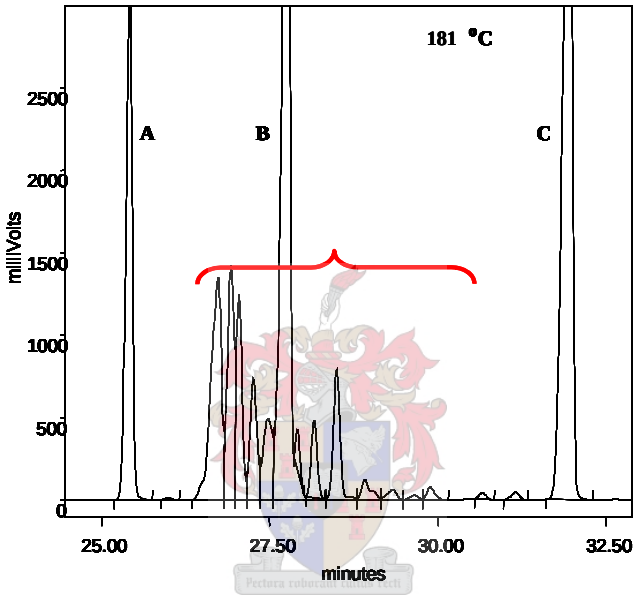



**Figure 23.2. Part of the GLC chromatogram of sample D showing the 18:0 (A), 18:1(B) and 18:2 (C) fatty acids, as well as the *cis* and *trans* 18:1 fatty acid isomers (under the bracket)**

The results of the eighteen margarines and spreads illustrated that the selected group of South African margarines had a lower *trans* fatty acid content than the margarines from some other countries. For the evaluation of the total *trans* fatty acid consumption of the South African population, the *trans* fatty acid content of the other staple foods also need to be determined.

# CHAPTER 5

# MATERIALS AND METHODS FOR CE

## 5.1 Introduction

Fatty acids are usually determined by GLC. The methods incorporate derivatisation to obtain volatility and detectability. The analytical time is usually long, because a very long capillary column is needed for the identification of *cis* and *trans* fatty acids. Alternative methods providing quicker analysis, preferably without a transmethylation step, are sought. A good alternative method is CE that generally provides high efficiency and fast analyses.

Recently CE has been contemplated for the analyses of fatty acids in oils and fats, and several approaches have been implemented towards such purpose (de Oliveira *et al*., 2003). Separation is generally conducted in the zone electrophoresis mode, under indirect UV detection, using a chromophore. It is also common practice to use large amounts of organic solvents in the buffer, to enhance fat solubility. MEKC is an alternative mechanism for the separation of fatty acids. For the separation of the *cis* and *trans* fatty acid isomers in margarines, it was decided to use MEKC with direct UV detection and sodium dodecyl sulfate (SDS) as the surfactant (Bohlin *et al*., 2003).

## 5.2 Samples

For the initial evaluation, a mixture of standard cis and *trans* FAMEs were used. (Nu- Chek-Prep, INC. Elysian, Minnesota, USA). The standard was diluted in 99.5% ethanol and running buffer was added. Finally, the mixture was degassed and mixed in a sonication bath.

## 5.3 Reagents and solutions

Sodium dodecyl sulfate, acetonitrile and boric acid were obtained from Aldrich (Sigma-Aldrich (Pty) Ltd, PO Box 10434, Aston Manor 1630, South Africa) and urea from Sigma (Sigma Chemical CO. St. Louis, MO 63178 USA). All the reagents were of analytical grade. Stock solutions of electrolytic components were prepared by dissolving appropriate amounts in Milli-Q water. Boric acid was adjusted to pH 9.2 with 20 M NaOH. The running buffer was prepared with 24 mM SDS, 20% acetonitrile, 40 mM boric acid and 4 M urea in milli-Q water. Prior to use, all the solutions were filtered through a 0.45 µm polypropylene filter.

## 5.4 Instrumentation

The experiment was conducted on a Hewlett-Packard CE system (Waldbronn, Germany), equipped with a diode array detector and a temperature controlled capillary cartridge. Detection was done with direct UV. The wavelength was set to 268 nm (Bohlin *et al*., 2003). An uncoated 58 cm fused silica capillary, with 50 cm effective length, and 50 µm internal diameter was used. The capillary was conditioned for 20 minutes with 2 M NaOH, 20 minutes with 0.1 M NaOH and 10 minutes with Milli-Q water. Between runs, the capillary was flushed for 2 minutes with 0.1 M NaOH, 2 minutes with organic modifier, 3 minutes with water and 3 minutes with running buffer. Samples were injected by applying a pressure of 50 mbar for 10 seconds. The temperature was set to 15$^{o}$C and the analysis was done with an applied voltage of + 30 kV.

# CHAPTER 6

# CE RESULTS AND DISCUSSION

The FAMEs analysed are very hydrophobic and consists of three *cis* and three *trans* 18:1 fatty acid isomers that differ only in position and geometry of the double bonds. This makes the separation difficult without the presence of a pseudostationary phase in the running buffer. SDS, as the surfactant, was tested as the pseudostationary phase. To enhance the solubility of the hydrophobic fatty acids, urea was added to the running buffer. Without urea, the fatty acids will precipitate (Bohlin *et al.,* 2003). No separation was achieved using 24 mM SDS and the SDS concentration was increased to 35 mM. A small peak was detected, but no separation. Since no baseline separation was achieved with different concentrations of SDS buffer, a longer capillary was utilised. Theory predicts that a longer capillary leads to longer migration times, and hence, band broadening because of longitudinal diffusion. The small peak did indeed elute much later, but without separation. It was expected that at least two peaks, a *cis* and a *trans* peak would be separated.

After the initial experiments, it was concluded that the separation of *cis* and *trans* 18:1 fatty acid isomers will not be possible with only one surfactant. Therefore, the use of different surfactants need to be investigated. The detection was done by direct UV detection, but indirect UV detection with chromophores also needs to be investigated further. The standard FAME mixture that was used comprises long chain fatty acids that are poorly soluble in aqueous solutions and the use of different non-aqueous buffers need to be investigated. Erim *et al*. (1995) reported that the longer chain fatty acids dissolve considerably better with a buffer containing 75% acetonitrile. Under these conditions, fatty acids with chain lengths up to 19 carbons could be completely separated.

Capillary electrochemistry (CEC) is an interesting alternative to the normal CE. In CEC, the mobile phase flow is generated electrokinetically by using a high voltage. Instead of the fused silica open tube capillary used in CE, a capillary packed with a stationary phase is used for selectivity. CEC combines the efficiency and analytical speed of CE with the selectivity GLC.

The use of CE methodologies, for the identification and quantification of the different *cis* and *trans* fatty acid isomers in partially hydrogenated oils, is a possibility, but plenty more research is needed. As the standardisation and optimisation of this technique fall outside the scope of this study, the comparison between the two methodologies could not be done.

# CHAPTER 7

# CONCLUSIONS

The first important step in the identification and quantification of the different *cis* and *trans* fatty acid isomers in food samples, is the correct extraction of all the fatty acids. Of the three different extraction solutions evaluated in this study, it can be concluded that the chloroform/methanol (2:1) solution gave the best fatty acid recovery. Therefore, this solution can be recommended for the extraction of the fatty acids in margarines.

Before the extracted fatty acids can be analysed by GLC, it is necessary to convert them to low molecular weight non-polar derivatives, such as methyl esters. In this study, two transmethylation reagents were evaluated and it was found that a solution of 5% concentrated sulphuric acid in double distilled methanol gave a 98.5 ± 0.35% FAME recovery using a 120 m capillary column. The FAME recovery was about 7% lower when using a 0.5 M sodium methoxide in methanol solution, and it was concluded that there was some sample loss or incomplete transmethylation when using 0.5 M sodium methoxide in methanol. A recovery loss was also observed when the ratio of sample concentration to volume of transmethylation reagent increased. When working with samples of unknown fatty acid concentration it is recommended that an acid-catalysed transmethylation solution be used.

The column length plays a critical role in the analyses of *cis* and *trans* isomers in the samples. Although a 30 m BPX 70 column gave good separations with a short analytical time for routine sample analyses, the *cis* and *trans* fatty acid isomers in the samples, especially those occurring between 18:0 and 18:2, could not be separated. Even the use of different column temperatures and column gas flow rates did not improve separation. The analytical elution range of the different *cis* and *trans* isomers of 18:1 were contracted into a too narrow retention time range for proper identification.

The 120 m BPX-70 column provided the required mechanism for extending the retention times of the different isomers by retaining the different compounds longer. In this way, the

retention times of the different isomers were pulled apart, and a greater separation space was available to the different isomers.

Different column temperatures have a major impact on the separation power of the column. Isothermal operation at 181$^{o}$C produced the least overlapping peaks, but some of the isomers will always overlap using a BPX-70 column regardless of the column temperature you use. Isothermal operations above or below 181$^{o}$C produced some additional isomer overlapping problems.

Except for a change in the retention times, there was very little effect on the resolution and column efficiency with the different gas velocities when hydrogen was utilised. Therefore, the precise flow rate was less critical. Although hydrogen is highly flammable, it remains the best carrier gas to use for the analyses of fatty acids because of its high diffusivities and low resistance to mass transfer. Even when using nitrogen or helium as a carrier gas, hydrogen is still needed for the FID flame.

The labelling law specifies that only the total concentration of the *trans* fatty acids must be displayed on the food label, though by using a long highly-polar capillary column, the different *cis* and *trans* isomers could be resolved making it possible to identify and quantify most of the isomers. From the concentrations of the different isomers, a very good prediction can be made of the source of the oils and fats that were used in the preparation of the margarines.

The Ag-TLC fractionation of the samples into the *cis* and *trans* 18:1 fatty acid isomers remains a good method prior to GLC analyses, to identify the overlapping *cis* and *trans* isomeric groups. However, this method did not separate all the different overlapping isomers. Two negative aspects of this method are the difficulty to quantify the different isomers, and the very laborious technique. With TLC, there is always a possibility of loosing some of the fatty acids during the long and laborious analytical preparation steps.

The results of the different margarines analysed, were surprising. Of the 18 samples analysed, only one had a high *trans* fatty acid concentration. Overall, local margarines do not have *trans* fatty acid content as high as that reported for some other countries. Although the *trans* fatty

acid content of the selected group of margarines was low, the total saturated fatty acid content was higher than the recommended percentage content. In order to calculate the total intake of *trans* fatty acids more data is needed, particularly on the *trans* fatty acid content of fast foods and other baked products made from shortenings. An advantage of using GLC fitted with a long highly-polar capillary column to analyse foods prepared from partially hydrogenated oils and fats, is that all the normal occurring and most of the *cis* and *trans* fatty acids in the samples can be identified and quantified in one analytical run.

An accurate determination of *trans* fatty acid intakes of individuals will only be possible after food composition tables have been updated with the *trans* fatty acid content of the different foods consumed in our country. This information is essential to study the effect of *trans* fatty acids in epidemiological studies, and to explore the potential negative effects specific *trans* isomers in certain foods have on different diseases.

In conclusion, this study demonstrated that the major *cis* and *trans* isomers can be identified and quantified by GLC using a 120 m BPX-70 capillary column. Although these capillary columns improved the separation, not all overlapping isomers could be separated in one isothermal column temperature run. To identify more of the overlapping isomers, experiments with different types of columns should also be conducted.

# CHAPTER 8

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