

**QUALITY MANAGEMENT AND FUNGAL
TRANSFORMATION IN THE EDIBLE OIL INDUSTRY**

by

MANJUSHA JOSEPH

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Department of Microbial, Biochemical and Food Biotechnology
Faculty of Natural and Agricultural Sciences
University of the Free State
Bloemfontein 9300
South Africa

Promoter: Prof. J.L.F. Kock
Co-promoters: Prof. B.C. Viljoen
Dr. E. Van Heerden
Dr. C.H. Pohl
Dr. A. Hugo

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This PhD thesis is dedicated to:

My late **DAD** (*Joseph*)

Chapter 1

Introduction

1.1 Motivation

Approximately 100 000 tonnes of edible frying oil and fat waste, mainly derived from sunflower oil, is produced each year from the estimated 54 000 frying establishments in South Africa (Pelesane *et al.*, 2001). Many of these establishments overuse or abuse their oils to save money. Such practices may result in oil breakdown and the production of harmful compounds, which can cause diseases such as cancer and diarrhoea when consumed (STOA Report, 2000; Anelich *et al.*, 2001; Kock *et al.*, 2002; Haw, 2003).

As a result, approximately 30 % of frying oil and fat waste in S.A. (Prof JLF Kock, University of the Free State, Personal communication, 2004) can be regarded as unhealthy while the other can be considered still useful for human consumption containing within S.A. regulatory limits i.e. equal to or less than 16 % polymerised triglycerides (PTGs) and/or equal to or less than 25 % polar compounds (PCs) (Kock *et al.*, 1996; Kock *et al.*, 1999; Kock, 2001; Kock *et al.*, 2002; Haw, 2003).

In order to ensure that only oils and fats fit for human consumption are used during frying processes in S.A., it is important that sound quality control procedures are applied to all sectors of the oil industry. This is of special significance when taking into consideration the numerous cases of frying oil misrepresentation, adulteration and overuse reported over the years in S.A. (Kock *et al.*, 2002).

The available 70 % of oil and fat wastes that are still within the regulatory limits and therefore considered as safe, have the potential to be used for the processing of usable

foodstuffs and biotechnologically important products, especially since these wastes can be considered as cheap (zero cost) and high energy substrates (Kock *et al.*, 2002). The ability of these wastes to serve as substrate for high value lipid production has been reported. Jeffery *et al.* (1997) showed that fat and oil wastes can be transformed to high value lipids such as gamma-linolenic acid (GLA) in the presence of sodium acetate. Plant oil containing GLA such as evening primrose oil (EPO) is currently in use in the cosmetic, food and pharmaceutical industries for nutritional and pharmaceutical preparations (Christie, 1999).

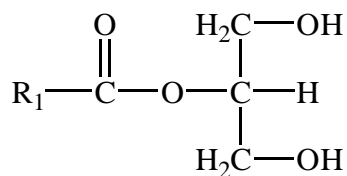
Thus, the aim of this study became to investigate (1) the quality control procedures currently in place in the main areas of the frying oil industry to combat misrepresentation and adulteration practices and (2) to further investigate the production of high value lipids such as EPO equivalents (EPOeq) from safe used frying oil and fat wastes.

1.2 Frying fats and oils

Fats and oils (henceforth referred to as oils) are bulk storage materials which are produced by plants, animals and microorganisms (Frankel, 1998). These compounds are also known as lipids, which are insoluble in water but soluble in organic solvents such as chloroform, alcohols and ethers. They are concentrated sources of energy (*ca.* 9kcal/g), serve as a supply of fat-soluble vitamins, contribute significantly to the feeling of satiety and also render food more palatable (Chow & Gupta, 1994; Frankel, 1998). In this thesis, emphasis will be placed on frying oils i.e. those that are used for the frying of various foods.

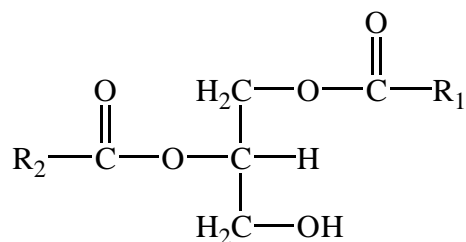
Frying oils contain mainly triacylglycerols (TAGs) (Frankel, 1998). Small amounts of other lipids such as monoacylglycerols (MAGs), diacylglycerols (DAGs), phospholipids (PLs) and free fatty acids (FFAs) are also found (Figure 1). These compounds are defined as fats or oils depending on whether they are solid or liquid at room temperature (Erickson, 1996; Ratledge & Wilkinson, 1988).

Monoacylglycerol

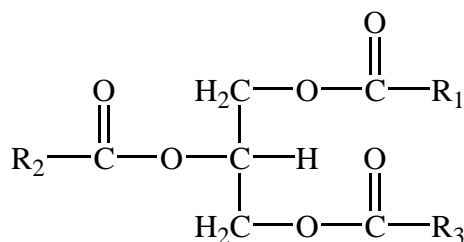
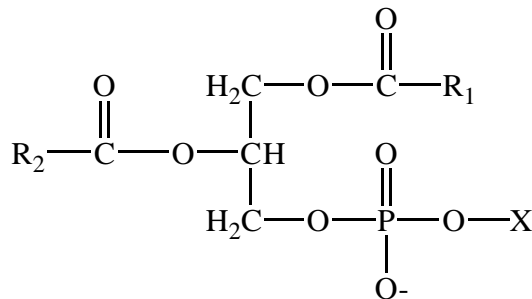


1-Acyl-*sn*-glycerol

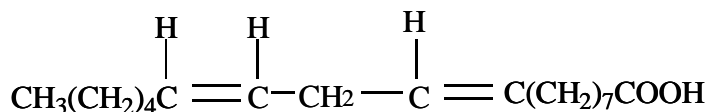
Diacylglycerol



1,2-Diacyl-*sn*-glycerol

Triacylglycerol1,2,3-Triacyl-*sn*-glycerol**Phospholipid**

Phosphatidic acid

Free fatty acid

Linoleic acid (C18:2)

Figure 1. Structures of fatty acid derivatives. R₁ CO-, R₂ CO-, R₃ CO- represent fatty acyl groups (Ratledge & Wilkinson, 1988). X = different ligands can be esterified at this point i.e. hydrogen, choline, serine, etc.

1.2.1 Composition of frying oils

Many plants are currently in use for the production of edible oil and about 40 different oilseed crops have been described (Shukla, 1994). However, out of these, only 10 are edible and of commercial value. Seven of these are seed crops, namely cotton seed, groundnut, rape seed, safflower seed, sesame seed, soybean and sunflower seed. The remaining three are tree crops and include coconut, olives and palm oil (Shukla, 1994).

These edible oilseeds and tree crops account for about 70 % of the world's edible oil production. The remaining 30 % is animal fat, which includes fish oils (2 %). Of the total fats and oils produced, about 80 % is consumed by humans, 6 % is used as animal feed and 14 % is distributed to the oleochemical industry (Shukla, 1994).

In South Africa, approximately 350 000 tonnes of vegetable oils are used in total per year in the food industry with sunflower oil being the most consumed oil followed by imported palm olein (Bareetseng, 2000). Annual consumption of sunflower oil is 155 000 tonnes at a price of approximately R5600 per tonne. The high usage of sunflower oil in South Africa is due to its availability and low price (Prof JLF Kock, University of the Free State, Personal communication, 2001).

Fatty acids of plant and animal origin contain an even number of carbon atoms in straight chains with a terminal carboxylic group and may be fully saturated (containing no double bond), mono-unsaturated (containing one double bond) or polyunsaturated (containing two or more double bonds). Table 1 shows the fatty acid (FA) composition of some of the important oils. In this table, the oils have been grouped according to the number of bonds they contain i.e. saturated, mono-unsaturated or polyunsaturated. Butterfat, beef tallow and lard (Table 1) are examples of saturated animal fats. Most of the edible plant oil crops contain large amounts of mono- and/or polyunsaturated fatty acids (PUFAs) (Shukla, 1994).

Oil or fat	4:0	6:0	8:0	10:0	12:0	14:0	16:0	18:0	20:0	16:1	18:1	20:1	18:2	18:3	S	M	P
Saturated																	
Beef tallow						3	24	19		4	43		3	1	46	47	4
Butterfat	4	2	1	3	3	11	27	12		2	29		2	1	63	31	3
Cocoa butter							26	35	1		35		3		62	35	3
Coconut oil		1	8	6	47	18	9	3			6		2		92	6	2
Lard						2	26	14		3	44	1	10		42	48	10
Palm kernel oil	1	1	3	4	48	16	8	3			15		2		83	15	2
Palm oil						1	45	4			40		10		50	40	10
Mono-unsaturated																	
Olive oil							13	3	1	1	71		10	1	17	72	11
Peanut oil							11	2	1		48	2	32		14	50	32
Rapeseed oil							4	2			62		22	10	6	62	32
Polyunsaturated																	
Corn oil							11	2			28		58	1	13	28	59
Cottonseed oil					1		22	3		1	19		54	1	26	20	55
Safflower							7	2			13		78		9	13	78
Soybean oil							11	4			24		54	7	15	24	61
Sunflower							7	5			19		68	1	12	19	69

4:0=butyric acid; 6:0=caproic acid; 8:0=caprylic acid; 10:0=capric acid; 12:0=dodecanoic acid; 14:0=myristic acid; 16:0=palmitic acid; 16:1=palmitoleic acid; 18:0=stearic acid; 18:1=oleic acid; 18:2=linoleic acid; 18:3=alpha-linolenic acid; 20:0=arachidic acid; 20:1=eicosenoic acid; S=total saturated fatty acids; M=total mono-unsaturated fatty acids; P=total polyunsaturated fatty acids

1.3 Manufacturing of frying oils

1.3.1 Extraction

A crude oil has to be extracted from animal or vegetable tissue before it can be refined. A rather simple process called rendering is used to extract animal fats. Rendering may be divided into two categories: dry and wet. Dry rendering involves heating of fat-containing tissue, which results in the solidification of the proteinaceous material and release of fat. Wet rendering consists of using steam under pressure to cook the fat-containing tissue thereby producing an edible protein as well as edible fat. The FFA content of the fats reflects their quality. A high FFA content indicates either errors in the processing of the fat or mishandling of the raw materials (Erickson, 1996).

The oil from oilseeds is obtained by either mechanical pressing, also known as pressure extraction, or by solvent extraction. Figure 2 shows a flow diagram of the mechanical extraction process. The process involves decortication of seeds, grinding or flaking of seeds to reduce size, followed by heating. The preparation of seeds is a very critical step as it is essential that the moisture level of the seed must be less than 5 % and the material must be cooked enough to permit ready release of the oil. Mechanical extraction has a number of drawbacks such as low yield, high power requirements as well as high maintenance. With solvent extraction (Figure 3), a suitable solvent is used to dissolve the fat from the fat containing tissue. Again, the preparation of oil seeds for solvent extraction is an important step. Though the principle remains the same, current continuous processes are quite complex and require large capital investments. A generic oil seed extraction flow diagram is indicated in Figure 3 (Perkins & Erickson, 1996).

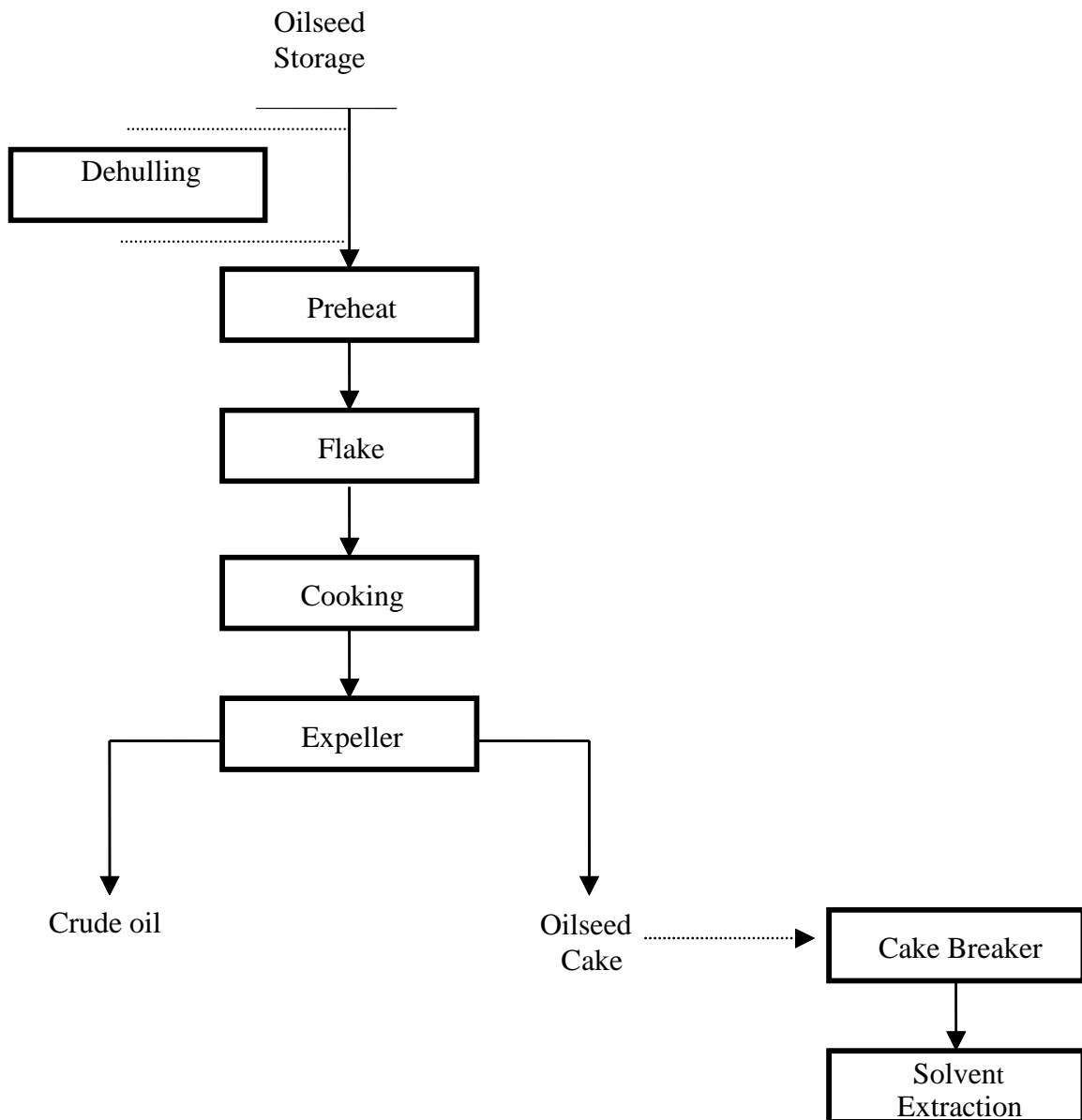


Figure 2. Expeller process (Perkins & Erickson, 1996).

Hexane is the most commonly used solvent in the extraction process to obtain crude oil. Once the crude oil is obtained, it is desolventized and toasted to obtain the oilseed meal.

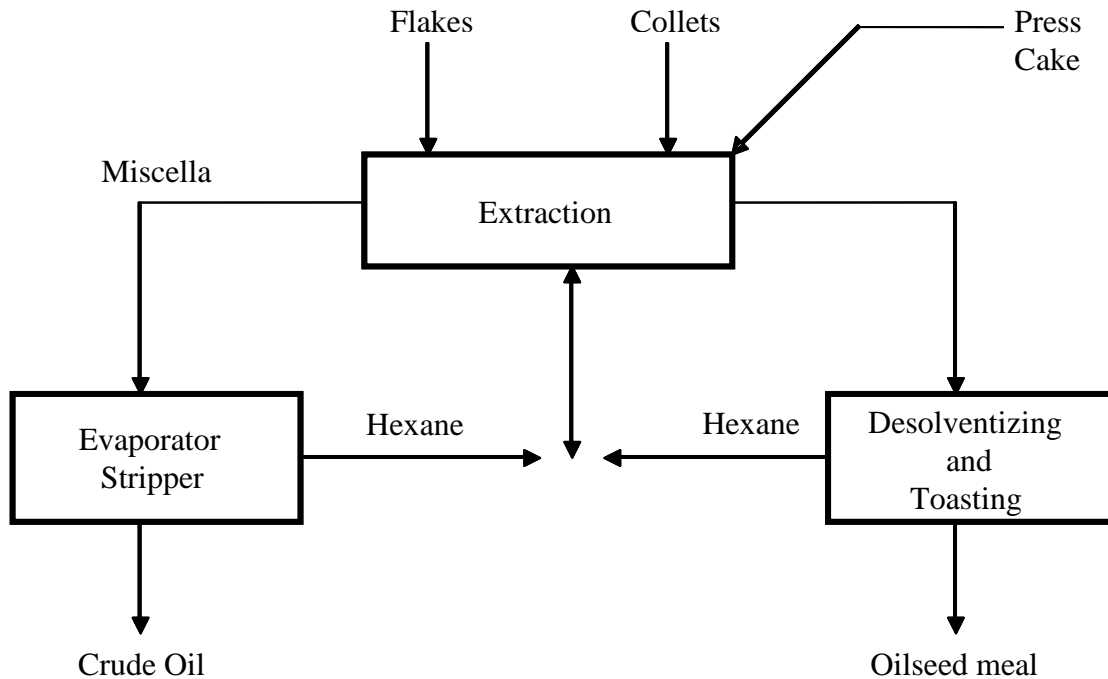


Figure 3. Solvent extraction process (Perkins & Erickson, 1996).

1.3.2 Refining of crude oils

The removal of unwanted constituents from crude oils is referred to as refining. The quality of an oil depends on the raw material used and the methods used for extraction. Figure 4 is a flow diagram of the steps involved in the refining of oils (Perkins & Erickson, 1996).

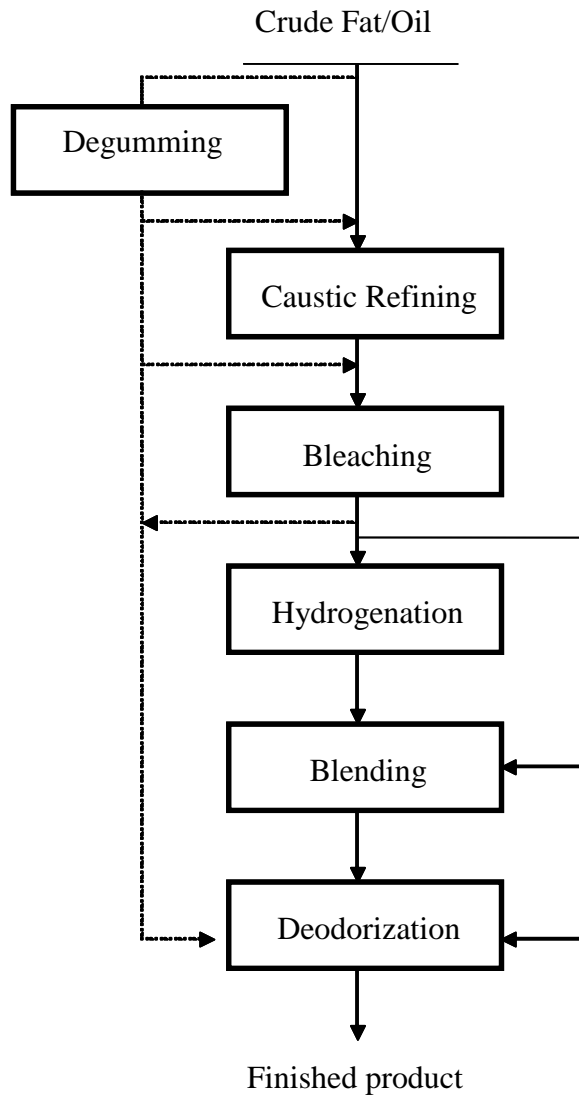


Figure 4. Generic oil refining flow diagram (Perkins & Erickson, 1996).

1.3.2.1 Degumming

The hydration of phosphatides present in oil by addition of water followed by centrifugation, is known as degumming. Non-triglyceride components such as residual meal, metal fragments, and other insolubles are removed from the crude oil by this process. Properly filtered and degummed oil is clear with less than 20 ppm phosphorous present (Erickson, 1996; Orthoefer & Cooper, 1996).

1.3.2.2 Caustic refining (Neutralization)

Caustic refining is carried out in order to remove FFAs from the oil. A predetermined amount of sodium hydroxide is mixed with the oil, followed by heating. This allows the sodium hydroxide to react with FFAs to form water soluble soaps which are removed by centrifugation (Erickson, 1996; Orthoefer & Cooper, 1996).

1.3.2.3 Bleaching and adsorption treatment

Bleaching is an adsorption process which is performed to remove colour bodies, residual soaps and phosphatides. Properly bleached oils provide maximum flavour stability in finished products (Erickson, 1996; Orthoefer & Cooper, 1996).

1.3.2.4 Deodorization

This is a steam distillation process carried out under vacuum (1 - 5 mm Hg) and at temperatures ranging from 210 - 270 °C. The purpose of deodorization is to obtain a flavourless product with an FFA content of less than 0.05 % for frying oils. After deodorization, antioxidants such as tertiary butyl hydroquinone (TBHQ), butylated

hydroxy aniline (BHA), butylated hydroxy toluene (BHT), ascorbyl palmitate and tocopherols may be added to enhance stability (Erickson, 1996; Orthoefer & Cooper, 1996).

1.3.2.5 Hydrogenation and formulation

The addition of hydrogen to the double bonds of unsaturated FAs attached to the triglycerides is known as hydrogenation and is used widely in the production of margarine. The purpose of carrying out hydrogenation is to increase the oxidative stability of an oil as this is very important for frying purposes. The extent of hydrogenation is determined by the Iodine Value (IV), which is defined as the grams of iodine that combine with 100 g of oil (Erickson, 1996; Orthoefer & Cooper, 1996).

1.3.2.6 Blending (Winterization)

Blending is carried out to remove high-melting triglycerides from lower-melting components. During this step, oil is slowly cooled to force crystallization of higher-melting glycerides. The crystallized components are removed by filtration which results in a clear fluid at room temperature (Erickson, 1996; Orthoefer & Cooper, 1996).

1.4 The frying process

Deep oil frying is a popular method of cooking which is commonly used for the manufacture and preparation of foods (Croon *et al.*, 1986; Al-Kahtani, 1991; Lin *et al.*, 2001). Fried food is a major item in the diet of many people in South Africa (especially those that fall in the lower income bracket) despite concerns about calories, cholesterol as

well as oil intake (Kock *et al.*, 1995; Kock *et al.*, 1996; Anelich *et al.*, 2001). The oil used for frying often determines the acceptability of food prepared in it. Although the frying oil primarily serves as a heat exchange medium, the oil makes up a significant portion of the final food product and often determines the acceptability of food prepared in it (Al-Kahtani, 1991; Orthofer & Cooper, 1996).

Many factors such as type of frying oil, frying temperature, frequency of changing the oil, design and material of the frying equipment and cleaning of the fryer are very important for the quality of the deep fried food products. If such factors are not under continuous control, deep frying will result in food that is of bad quality (Croon *et al.*, 1986; Al-Kahtani, 1991; Orthofer & Cooper, 1996). Both physical and chemical changes occur in oil as a result of frying. A variety of reactions such as oxidative and hydrolytic degradation occur in the oil and numerous decomposition products are formed. Highly abused or overused oils contain oxidized and polymerised materials, which not only affect the quality of the fried food but may be harmful to human health (Croon *et al.*, 1986; Al-Kahtani, 1991; Anelich *et al.*, 2001).

1.4.1 Changes that occur in oils during the frying process

During the frying process (Figure 5), food is submerged into the frying oil, which is heated to temperatures as high as 200 °C. This leads to several changes resulting in the breakdown of the oils and inactivation of anti-oxidants. Firstly, the moisture from the food escapes as steam and comes into contact with the oil, hydrolysing the ester bonds of the triglycerides resulting in the formation of FFAs, DAGs, MAGs and glycerol.

Monoglycerides and FFAs are emulsifiers, which are promoters of fat hydrolysis. Micellization of water keeps MAGs and FFAs in the fat phase for a longer time, thus further increasing hydrolysis (Fritsch, 1981).

The heavy metals originating from the food and the added spices cause the FFAs to transform to soap compounds causing foaming on the surface of the frying oil. Foaming results in an increase in oil aeration and hence an increase in oxidation (Anelich *et al.*, 2001). This may lead to the formation of hydroperoxides, which could undergo fission to produce alcohols and aldehydes, dehydration to produce ketones and the formation of free radicals to produce dimers, trimers, epoxides and other compounds. Heating oils at higher temperatures leads to the formation of cyclic compounds (Kock, 1998). The oxidation of oils depends largely on the degree of unsaturation of the FAs. Linoleic acid (18:2), which is a highly unsaturated fatty acid and therefore most susceptible to oxidation, is the major component of frying oils such as sunflower, soybean and corn oils (Anelich *et al.*, 2001). Oils can be oxidized via autoxidation, thermal oxidation, enzymatic oxidation or photo-oxidation (Chow & Gupta, 1994; Frankel, 1998). In this section, emphasis will be placed on autoxidation since it occurs readily during the frying process.

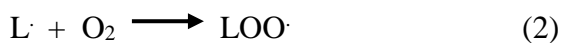
1.4.1.1 Autoxidation

Autoxidation is the process of oxidation at room temperature and/or elevated temperatures. It can be divided into three phases, namely initiation, propagation and termination. During initiation, hydrogen is abstracted from the α -methylene carbon of

FAs to yield a free radical (Equation 1). A free radical initiator or catalyst is needed for the reaction to take place (L = fatty acid).



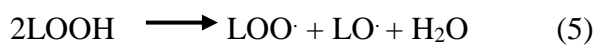
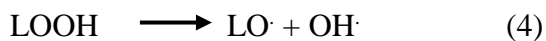
Once a free radical is formed, peroxy radicals may be formed through reaction with atmospheric oxygen (Equation 2).



Free radicals may also abstract hydrogen from other unsaturated molecules to form a hydroperoxide (LOOH) and a new free radical (Equation 3).



The free radical reaction can be accelerated and propagated by chain branching (donation of hydrogen atoms to lipid peroxy radicals) of hydroperoxides, to generate even more free radicals (Equations 4 and 5). Free radicals formed can initiate fatty acid oxidation at a faster rate. Once initiated, the free radical reaction is self-sustaining and is capable of oxidizing large amounts of lipids.



The chain reaction may be terminated by antioxidants such as vitamin E (tocopherols) that react with a peroxy radical and results in the removal a free radical from the system (Equation 6).



Also, the chain reaction may be terminated by self-quenching or polymerisation of free radicals to form non-radical dimers, trimers and polymers (Equation 7).



Oxidation products of oils may be broken down to form smaller organic compounds such as aldehydes, alcohols and acids. This breakdown is catalysed by high temperature and the presence of transition metals such as iron and copper that enter foods via water or spices used in food preparation. A large number of FA oxidation products such as carbonyls, alcohols, esters and hydrocarbons are produced via cleavage reactions (Kock *et al.*, 1995).

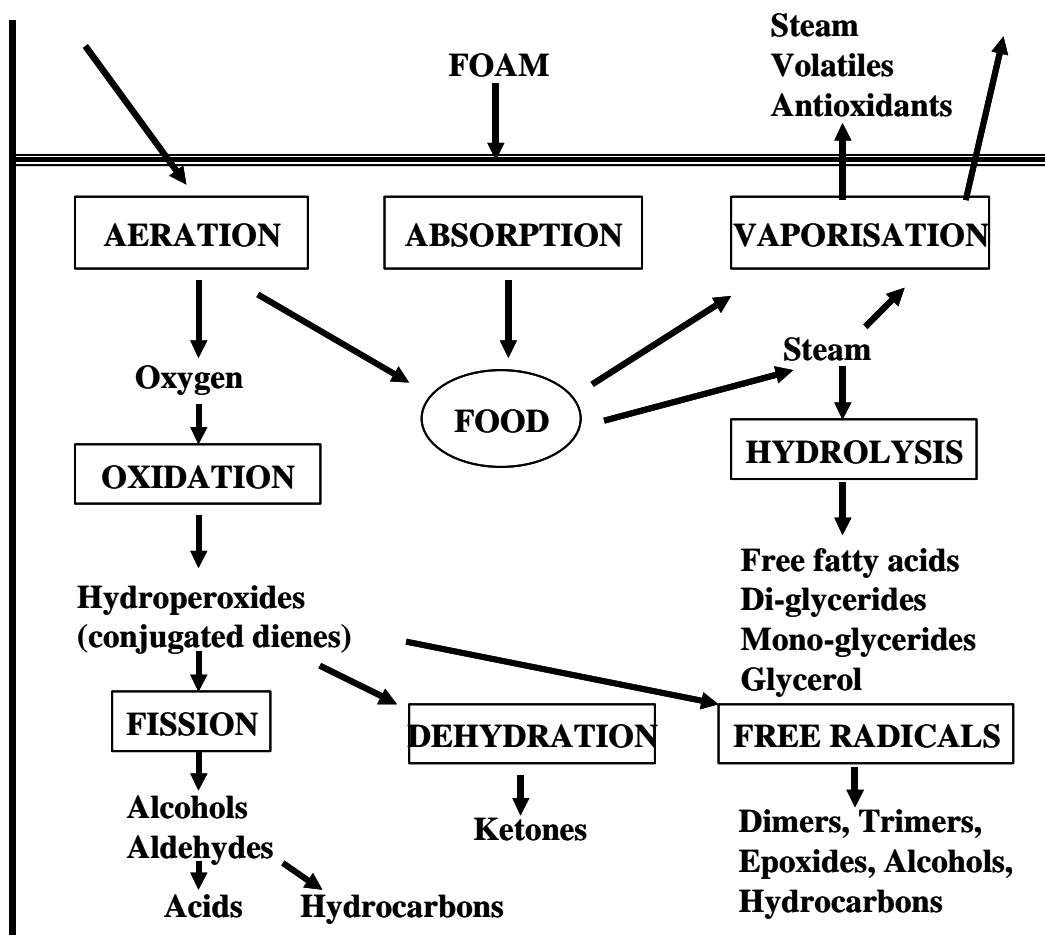


Figure 5. Changes that occur during deep oil frying (Fritsch, 1981).

1.5 The abuse of frying oils in South Africa

Many frying establishments in South Africa abuse their oils during frying in order to save money. Abuse of oils fall under two categories, adulteration and over-oxidation. Adulteration can be defined as the addition of mineral oils and other oils in order to increase oil volume and usage. The first report on adulteration practices was in 1938

from Durban, where several people became ill after consuming contaminated table oil. Since then, many other cases were reported where addition of torpedo oil in Germany in 1940, machine gun oil in Switzerland in 1944 and helicopter oil in Vietnam in 1970 occurred, leading to many people becoming ill after consumption. More than 20 000 people became ill while 600 died in Spain in 1981 due to the consumption of adulterated rapeseed oil that was believed to be olive oil (Mitchell, 1987; Kock, 1998; Kock *et al.*, 1999).

The interest in abused oils in South Africa started when a used restaurant oil refining company claimed that it could refine old, dark used restaurant oil destined for pig farms, to oil perfect for frying and even better than unused oil. This is impossible, since the triglycerides which are the major components of frying oil cannot be restored using normal refining methods. Dark over-used oil was also refined using lime and bleach. This lighter oil was then used again in the frying of food (Anelich *et al.*, 2001).

A survey was carried out by the University of the Free State (UFS) in collaboration with the Bloemfontein Municipality in 1994. Fifty-four frying establishments in Bloemfontein were sampled by Environmental Health Officials (EHOs) without prior notice and these samples were analysed by UFS. Of these establishments 69 % were found to use oils containing high levels of polar compounds (PCs) i.e. > 25 % and 88 % of these establishments distributed these abused oils to informal sectors through staff members where it was further re-used and abused in frying procedures (Kock *et al.*, 1996; Kock, 1998; Kock *et al.*, 1999).

If strict quality control procedures are implemented by oil collectors at frying establishments, oils within the regulatory limits would be used in the preparation of foods at restaurants (Kock, 2001) and would therefore be safe for distribution to animal feed manufacturers to be incorporated into animal feed and thus the human food chain. The development and application of a scientifically based Statistical Process Control (SPC) system by animal feed manufacturers and oil collectors is highly necessary for the installation of an efficient quality control program in the restaurant oil industry (Pyzdek, 1998).

1.6. Effect of excessively used frying oils on the health of humans

Over-oxidation results in the formation of harmful breakdown products such as polymers, cyclic monomers, low molecular weight products of which examples include malondialdehyde, 4-hydroxyalkenals and oxidized fatty acids. These compounds lead to adverse biological and toxicological effects in mice and rats as well as in humans. These effects include loss of appetite, growth depression, diarrhoea, histological changes in tissues, tissue enlargement, interference with reproduction, cancer and arteriosclerosis (Chow & Gupta, 1994; Kock, 1998; Kock *et al.*, 1999; STOA Report, 2000; Anelich *et al.*, 2001).

1.7 Final regulations

Due to the abuse of frying oils in S.A. and the potential health risk of these oils, strict regulations under the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act 54 of 1972)

were published on 16 August 1996 prohibiting the use of adulterated and over-used frying fats in the preparation of food (Anelich *et al.*, 2001). The final regulations state the following: “for the purpose of section 2 (1) (b) (i) of the Act, in so far as it applies to foodstuffs, edible fats and oils used for the frying of food are hereby deemed to be harmful or injurious to human health, unless they contain equal to or less than 16 % polymerised triglycerides (PTG); and/or equal to or less than 25 % polar compounds (PC)”. Frying oils that do not comply with the set levels, may not be used in the preparation of food (Kock, 1998; Kock *et al.*, 1999).

1.8 Quality control procedures

Figure 6 shows a flow diagram of the network in the frying oil industry in S.A. The network starts with the farmers who supply seeds to the oil expressers for extraction and refining of oils. The refined oils from the oil expressers are then distributed to the frying establishments to be used for frying purposes. Used oils from the frying establishments are then collected by oil renderers and distributed to the animal feeds and oleochemical industries where these oils are used for incorporation into animal feed or used in industrial processes (making of soaps etc) respectively. Some of the oils collected by the oil renderers are distributed illegally to the informal sectors where it is further used (Kock *et al.*, 1999). S.A. oil processors produce oils that are comparable to the best in the world i.e. containing less than 3 % breakdown products (Oilseeds Advisory Committee Report, 2000). The present abuse of oils in S.A. is highly disturbing as oils are degraded by over-usage by some frying establishments to levels unheard of in other countries. Furthermore, these severely degraded oils which are not even fit for animal consumption

are distributed to poor communities at a low price where it is further used (Kock *et al.*, 1999; STOA Report, 2000; Kock, 2001). There have been indications that these deteriorated oils are also mixed with new unused oils and then sold as new. This is of concern since the more frying oils are degraded, the greater the chances of potentially toxic compounds to be formed and more of these oils to be absorbed by the fried food. Consequently, more of these unhealthy oils are consumed. There have been numerous reports on the fraudulent sales of mixtures containing mainly water and paraffin, as new unused frying oil (Kock *et al.*, 1999).

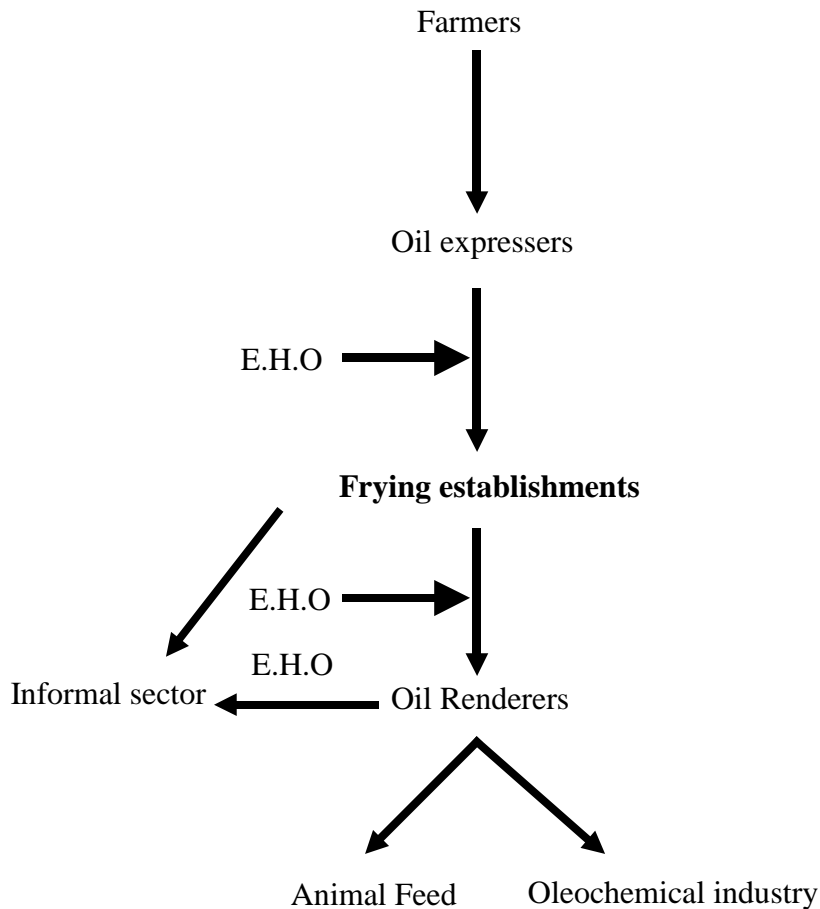


Figure 6. Flow diagram of the frying oil industry network in South Africa.

Due to malpractices and poor quality control (QC) systems used by restaurant oil collectors, many animal feed manufacturing companies show reluctance to incorporate these oils into their animal feed stocks. As an alternative, many restaurant oils are illegally recycled into the human food chain or sent to the oleochemical industry. The illegal route is preferred in S.A. since much higher prices are obtained compared to the legal oleochemical route. Since it has been estimated that more than 100 000 tonnes of used oils are available in S.A. per annum, the illegal distribution of over used oils for human consumption can be disastrous (Kock, 2001).

In order to install an efficient QC program in frying establishments, a sound scientifically based Statistical Process Control (SPC) system should be developed and applied by restaurants, restaurant oil collectors and animal feed manufacturers (Pyzdek, 1998). Inexpensive and rapid test kits are available for the quality control of restaurant oils. Quality control should be implemented to insure that only restaurant oils still fit for human consumption i.e. breakdown products (polymerised triglycerides) below 16 % are bought and incorporated into animal feed. In practice, smaller restaurant oil collectors (primary collectors) usually collect oil from frying establishments and deliver these to bigger oil collectors (secondary collectors). These oils are then distributed mainly to the oleochemical industries or to the animal feed manufacturers.

However, very few South African oil collectors have sufficient scientifically based QC systems in place to be able to select restaurant oils fit for inclusion into animal feed. In order to install an effective QC system for oil collectors, the smaller primary collectors

should be coordinated across S.A. to ensure that oils still within regulatory limits are collected. Oil wastes that are still within the regulatory limits and therefore considered as safe, have the potential to be used for the processing of usable food stuffs and biotechnological products, especially since these wastes can be considered as cheap (zero cost) and high energy substrates (Kock *et al.*, 2002).

1.9 Production of high value lipids using fungi

Even though the production of oils by fungi is well documented, no process has ever reached commercial realisation. This is mainly due to the high costs involved in biotechnological routes, which cannot compete against the low costs of agricultural seed oil production (Ratledge, 1991). Microorganisms are receiving increasing attention for their potential use in the oil industry, i.e. in the production of high value oils or for carrying out selected biotransformation reactions which may lead to higher value lipid products (Ratledge, 1991). Two major markets which oil from fungi or single-cell oil (SCO) may influence were identified and include the cocoa butter and gamma-linolenic acid markets (Roux *et al.*, 1994).

Gamma-linolenic acid (18:3) is an omega-6 essential fatty acid and is a precursor for the local hormones i.e. prostaglandins, leukotrienes and thromboxanes. A decrease in GLA production has been associated with various disease states such as diabetes, atopic eczema, premenstrual syndrome and coronary heart disease. Many clinical studies have shown improvement in patients with atopic eczema after administration of GLA rich oil (Raederstoff & Moser, 1992) and thus GLA is used in the treatment of eczema, diabetes,

premenstrual syndrome and other conditions. It is obtained from plants such as evening primrose, borage and blackcurrant (Horrobin, 1990). Cocoa butter is used largely in the manufacture of chocolate. The physical and chemical properties of cocoa butter play an important role in the melting behaviour, texture and mouth-feel of chocolate. Cocoa butter characteristics are dependent on the fatty acid ratios of approximately 30 % stearic acid (18:0), 30 % palmitic acid (16:0) and 30 % oleic acid (18:1).

Mucoralean fungi belong to the division Zygomycetes and contain economically important species used in the production of a wide range of organic compounds. Many studies have shown that carbon sources presented to fungi, especially to those in the genus *Mucor*, can influence fungal lipid composition such as gamma-linolenic acid (GLA) content. Consequently many mucoralean fungi were found to produce large amounts of GLA (Botha *et al.*, 1997). *Mucor circinelloides*, which is also known as *Mucor javanicus*, was used for the commercial production of GLA in the mid 1980s. The production plants were operated by J & E Sturge Ltd, Selby, Yorkshire, UK. The resulting oil had twice the amount of GLA as evening primrose oil but slightly less than borage oil. The oil had no observable toxicity and was sold commercially between 1985 and 1990. However, the plant closed in 1990 when the price of GLA oil fell from US \$ 50.55/kg to less than US \$ 25/kg. Idemitsu Ltd in Japan developed a similar process, using the filamentous fungus *Mortierella isabellina* for the production of GLA (Ratledge, 1994). This process is still in operation today.

Oleaginous micro organisms can accumulate 20 - 25 % oil and can use a wide variety of substrates of which most are sources of carbohydrates, including starch, ethanol, whey, peat and molasses (Ratledge, 1991). When oils are fed to fungi as growth substrate, lipases are produced which are responsible for the hydrolysis of mainly TAGs to FFAs and glycerol. These FFAs are then transported by diffusion into the cell where they can be metabolised either through beta-oxidation to yield energy or incorporated into the lipids of the fungal cells (Ratledge, 1991).

Jeffery *et al.* (1997) demonstrated a process by which sunflower oil fed to *Mucor* as a carbon source enhanced utilization of sunflower oil in the presence of sodium acetate, followed by doubling of fungal mass and an increase in the intracellular polyunsaturated GLA content when compared to growth conditions where sunflower oil was used as the sole carbon source. Later, Jeffery *et al.* (1999) demonstrated that it was not the sodium acetate *per se* that was responsible for the emulsification and the enhanced transformation of sunflower oil, but rather an effect of the rise in pH over the growth cycle. Pelesane *et al.* (2001) investigated the transformation of extensively used oils to GLA and reported that similar to what was observed with unused sunflower oil, the simultaneous metabolism of acetate markedly improved the conversion/biotransformation of used frying oil. This was due to the increase in pH, which favoured both emulsification of the oil and its cleavage by fungal lipase as well as their utilization for cell growth and production of cellular lipids.

The occurrence of significant levels of GLA in plant sources is rare and was first reported in 1949 for Evening Primrose Oil (EPO) (Lawson & Hughes, 1988). Over the last ten years, oil from the seeds of evening primrose (*Oenothera biennis*) has attracted special attention due to its unusually high GLA content (8 - 10 % by weight) (Lawson & Hughes, 1988; Schafer & Kragballe, 1991). Gamma-linolenic acid-containing oils are of considerable interest in the pharmaceutical industry as well as for use in dietary food supplements (Tsevegsuren & Aitzetmuller, 1993).

A decade ago, unknown about GLA containing oils was the distribution of GLA and other fatty acids in the triacylglycerol structure (Lawson & Hughes, 1988). The need to investigate the composition of TAGs is considered as essential since the therapeutic efficacy of the fatty acid containing GLA has shown to be dependent on the stereospecific structure of the TAGs rather than on the overall content of GLA itself (Cisowski *et al.*, 1993). A few years later, research carried out on native EPO and borage oil (BO) showed that GLA in both oils was distributed asymmetrically and was located preferentially at the *sn*-2 and *sn*-3 positions. It is known that the positional distribution of fatty acids on the glycerol moiety of TAGs can influence both the functional properties and the metabolism of fats and oils (Christie *et al.*, 1991; Angers & Arul, 1999). It has been shown that for peanut oil, the TAG structure can affect fatty acid absorption significantly. Stereospecific analysis usually starts with partial deacylation either by pancreatic lipase for the removal of a specific fatty acid or by a Grignard reagent (Angers & Arul, 1999). To determine the stereospecific analysis of TAGs, the procedures used are quite complex and involve chemical and enzymatic hydrolytic steps. Several

different methods are available to determine the positional distribution of TAGs such as, gas chromatography (GC), thin layer chromatography (TLC), chiral-phase or normal phase high-performance liquid chromatography (HPLC). The most appropriate method for the analysis of fatty acids, generally in the form of methyl ester derivatives is by GC (Redden *et al.*, 1995; Angers & Arul, 1999; Christie, 1999).

1.10 Purpose of Research

With this information as background the aims of this study became the following:

1. To investigate if current legislation was adequate to combat illegal practices (Chapter 2).
2. To develop bioprocesses for the biotransformation of oil wastes to EPO equivalents (Chapter 3).
3. To characterize EPO equivalents using Nuclear Magnetic Resonance (NMR) and Gas Chromatography (GC) analysis (Chapter 4).

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Chapter 2

Quality Management in the Frying Oil Industry

Parts of this chapter have been published in GRAIN SA., August 2002, 58-61.

1.1 Motivation

1.1.1 Frying oil industry in South Africa

The South African frying oil industry consists of seven sectors, namely the oilseeds producers, the oil expressers and refiners, frying establishments, oil collectors, animal feed and the oleochemical sectors. The network consists of approximately 16 oil extracting and refining companies, 50 000 frying establishments, 6 national oil collectors, 43 animal feed and 1 major oleochemical industries (Prof JLF Kock, University of the Free State, Personal communication, 2001). About 350 000 tonnes of vegetable oils are used in total per annum in the food industry in South Africa with sunflower oil (at R5600 per tonne) being the most consumed oil, followed by imported palm olein (Bareetseng, 2000). The availability and affordable price of sunflower oil in South Africa is responsible for its high usage especially in the frying industry (Bareetseng, 2000). In total approximately 100 000 tonnes of frying oil is discarded (i.e. mainly sunflower oil) annually in South Africa (Pelesane *et al.*, 2001).

1.1.2 Quality management (QM) procedures in the frying oil industry of South Africa

A survey was carried out to determine the quality control procedures currently in place in the frying oil industry in South Africa. Figure 1 shows the total process network where internal as well as external quality control (QC) procedures prescribed for the industry are indicated at each step. Imports are indicated in oval circles and local production is shown in squares. The first quality control point (QC1-Internal) concerns the oilseeds obtained from the farmers by the oil expressers and refining companies. Here, the protein, oil, moisture and fibre contents of the seeds are tested to ensure that the seeds adhere to a

good quality. The second quality control point (QC2-Internal) concerns the refining process. Different tests are done at each stage of the refining process to ensure that the final product is of high quality. On the crude oil received, tests such as refractive index (RI), free fatty acid content (FFA), colour, moisture, impurities, peroxide value (PV) etc. are carried out mainly as prescribed by the international American Oil Chemists Society (AOCS) and Codex Alimentarius. Free fatty acid content and soap content determinations are performed on the neutralized oil after washing. Bleached oil is tested for FFA, soap, PV, colour and phosphatides. Flavour, PV, FFA and colour are carried out on the deodorised oil and finally, before dispatch, solids, flavour and PV are determined. All these tests are performed according to the AOCS prescribed protocols (Prof JLF Kock, University of the Free State, Personal communication, 2001).

The third internal quality control point (QC3-Internal) is on the imported crude oil. The South African oil expressers import crude sunflower oil mostly from Argentina and palm oil from Malaysia. The imported crude oil is bought according to above specifications and Codex Alimentarius standards. The fourth quality control point (QC4-Internal) is on refined oil distributed to the frying establishments for use in the preparation of various foods. At this stage, the only QC measures carried out by frying establishments is buying oils that are of a reputable brand and believing the accompanying certificates of analysis. The fifth quality control point (QC5-External) is on imported refined oil which is bought according to a certain specification along with provision of an authentication certificate and distributed by oil expressers and refining companies to frying establishments. Here again, the only quality control carried out by the frying establishments is the knowledge

that the oils supplied are from a reputable supplier and believing accompanying analysis certificates. Frying establishments use mainly colour tests (such as Coltest - University of the Free State, Oxifrit test-Merck, Fritest-Merck, pH strips-3M) on the oil before it is discarded. The sixth quality control point (QC6-External) is monitored by oil collectors collecting used oils from frying establishments for distribution to either the animal feed or the oleochemical industries. According to the oil collectors, used oils that are not fit for animal feed are mostly sold to the oleochemical industries or distributed to chemical companies for various industrial applications at a much lower price. Oil collectors determine the quality of the waste oil to be distributed to the animal feed or oleochemical industries by determining its colour and breakdown levels using test kits such as the Oxifrit Test (Merck, Germany), Coltest (University of the Free State, Bloemfontein, S.A.) as well as 3M shortening (Food Service Business, U.S.A.) while many samples are sent to the lipid laboratory at the University of the Free State, (SA Fryer Oil Initiative - SAFOI) for quality analysis (Prof JLF Kock, University of the Free State, Personal communication, 2001).

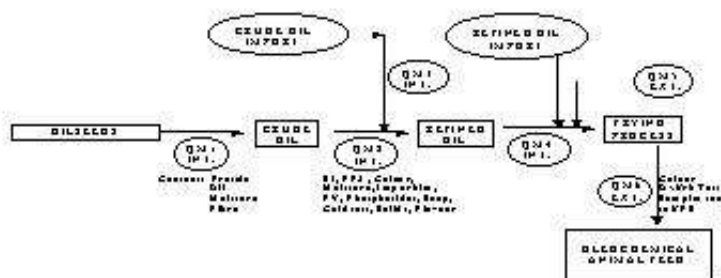


Figure 1. Total process network of the frying oil industry using various quality management procedures FFA= Free Fatty Acid, PV= Peroxide Value, QM= Quality Management, RI= Refractive Index, UFS= University of the Free State.

The main external QC points i.e. external (QC 5, 6), which are aimed at frying establishments and refined oils, are also performed by the Department of Health, which employs Environmental Health Officers (EHOs) to monitor the quality of refined and used oils obtained from frying establishments with the aim of law enforcement. The regulations followed are in accordance with the Codex Alimentarius standards and the Foodstuffs, Cosmetics and Disinfectants Act of 1972, which does not permit the usage of oils containing equal to and more than 16 % breakdown products for purposes of food preparation and the use of refined oils outside the Codex Alimentarius specifications.

1.1.3 Quality Management in relation to Codex Alimentarius and South African Regulations

From the surveys conducted (SAFOI, Kock *et al.*, 1999), South African oil expressers and refining companies have a good quality management system in place, where routine tests according to the internationally accredited methodology (AOCS and Codex Alimentarius) are conducted at each stage of the expressing and refining process to ensure that oils of high standard are produced. The problem with oil quality mainly starts at frying establishments where many overuse their oils until they become unhealthy. Many of these used oils are sold to the informal sector (poor) at a low price of R2500/tonne for further use. These unstable oils are then used repeatedly also in home frying practices thus resulting in the formation of high concentrations of breakdown products such as polymers, which are harmful to human health when consumed (STOA Report, 2000). The current food regulations under the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act 54 of 1972) of South Africa state the following “for the purpose of section 2 (1) (b) (i) of the Act, in so far as it applies to foodstuffs, edible fats

and oils used for the frying of food are hereby deemed to be harmful or injurious to human health, unless they contain equal to or less than 16 % polymerised triglycerides (PTG); and/or equal to or less than 25 % polar compounds (PC)". Frying oils that do not comply with the set levels may not be used in the preparation of food (Kock, 1998; Kock *et al.*, 1999).

However, in 1999, with all these QC protocols in place in the oil production and distribution industries (Figure 1), the SAFOI at the UFS exposed refined, fresh oils that contained high amounts of breakdown products i.e. sometimes up to 61 % PTGs. These oils were sold as new oil to the Tygerberg Hospital in the Western Cape (Kock *et al.*, 1999). During this time many similar cases were exposed (Kock *et al.*, 1999) where maximum deterioration levels were found in many oil brands sold in S.A. as well as the fraudulent sales of mixtures containing water and paraffin as new unused frying oils. These high breakdown values indicate the mixing of fresh unused oils with already used oils. An Oilseeds Advisory Board project (Kock *et al.*, 1999) has shown that refined oils produced by S.A. oil processors contain less than 3 % breakdown products and thus are comparable to the best in the world and any unused oil containing more than 3 % breakdown products is mixed. How could something like this happen in such a supposedly well-controlled network? Are the current prescribed QC procedures as well as Food Law (guided by the Codex Alimentarius) regulations inadequate to detect mixing practices? Should new regulations be included in the Food Law i.e. including measurement of breakdown products to effect this? Consequently, the aim of this chapter became to evaluate the sensitivity of the Codex Alimentarius guidelines and code of

practice to detect mixing practices and to determine if the current South African regulations prohibit the sale of oils already used or still in use for further application in food preparation.

1.2 Are the current South African food regulations sufficient to combat edible oil and fat abuse?

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The present regulations pertaining to the Foodstuffs, Cosmetics and Disinfectants Act, 1972; Act No. 54 of 1972 were found to be adequate in prohibiting any frying establishment to sell or distribute used oils and fats for re-use in the preparation of food – even after only one use! It is now possible to detect illegal mixing practices at low levels of used oil inclusion. Mixing practices as determined in unused oil samples in the past, have declined dramatically since 2000, and can be ascribed to the awareness campaigns of the SAFOI network as well as the Department of Health.

1.2.1 Introduction

Malpractices in South Africa are responsible for extensive degradation and adulteration* of frying oils and fats to levels unheard of in other countries. This happens notwithstanding the fact that the S.A. oil processors produce oils and fats that are comparable to the best in the world i.e. containing less than 3 % breakdown products (Oilseeds Advisory Committee Report, 2000). The above malpractices as well as the practise of misrepresentation** may not only compromise the health of consumers (giving cancer, diarrhoea, etc.), but will have a large negative impact on the sales of the S.A. oil processors as well as on the production of the oilseed producing farmers. It is estimated that these practices will deprive the S.A. edible oil and fat industry of more than 50 000 tonnes p.a. in refined oil production and sales. For an extensive review on this topic the reader is referred to Kock et al. (1999).

*Addition of used/unused oils and other compounds to edible oils in order, for example, to increase volume. **The sale of a product under a false name. This can occur through the blending or replacement of a particular oil or fat usually with less expensive oil or a mixture. For example, inexpensive sunflower oil is sometimes adulterated and sold as the more expensive olive oil.

To effectively combat this problem, effective regulations are needed. Up till now, many frying establishments in S.A. were of the understanding that the present regulations prohibit the sale and use of only over-used frying oils i.e. containing equal to or more than 16 % breakdown products also known as polymerized triglycerides (PTGs) (Foodstuffs, Cosmetics and Disinfectants Act, 1972; Act No. 54 of 1972). Consequently, it is assumed that oils and fats below regulatory limits and near the end of their usable life can be sold to anyone for further utilisation in food preparation. These oils are unstable and easily degraded beyond the set regulatory levels - even after a single use or after prolonged storage to produce hazardous compounds. This presumable “loophole” in the legislation is one of the main causes for the current malpractices. As a result, many frying establishments sell used frying oils and fats as standard procedure to poor communities and oil collectors for further use in food preparation. Some collectors even blend these oils and fats with new oils or attempt to clean these. These unstable mixtures are then resold as new to the public for use in food preparation. In many cases these oils are also sold to unsuspected animal feed factories for incorporation into animal feed (Kock et al., 1999).

In this study, the current regulations (including the Codex standards for purity and composition of edible fats and oils) under the Foodstuffs, Cosmetics and Disinfectants

Act, 1972 - Act No. 54 of 1972 (R1316; Government Gazette No. 17365) will be evaluated. In order to determine if these are sufficient, the aim of this study will be to determine if the current regulations prohibit the sale of oils already used or still in use for further application in food preparation. In addition, the question whether illegal mixing practices still occur in S.A. will be addressed.

2. Materials and methods

2.1 Evaluation of current regulations

In this study samples with different breakdown levels were compared to the regulations, based on Codex standards, included in The Foodstuffs, Cosmetics and Disinfectants Act, 1972 - Act No. 54 of 1972 (R1316), Government Gazette No. 17365*. This was done to evaluate if the present regulations are sufficient to detect low oil breakdown occurring after limited use or after any mixing practices i.e. resulting in oils and fats with above 3 % PTG breakdown products. A previous study has shown that normally refined unused oils in S.A. contain breakdown levels of equal to or smaller than 3 % PTGs (Oilseeds Advisory Committee Report, 2000).

2.1.1 Preparation of samples with different breakdown levels

A total of 18 samples with different concentrations of PTGs were prepared by diluting an over-used oil containing 23.01 % PTGs with unused oil containing only 1.26 % PTGs. The data are indicated in Figure 1. All experiments were performed in triplicate. Polymerised triglycerides were determined as prescribed by legislation [regulations under

the Foodstuffs, Cosmetics and Disinfectants Act, 1972 (Act 54 of 1972) - published on 16 August 1996] (Beljaars et al., 1994).

* G.N. R1316 of 16 Aug. 1996 Reg. 2(4): “The standards for the purity and composition of edible fats and oils shall be (if any) those laid down in the latest edition of the *Codex Alimentarius Standards for Fats and Oils* or the *British Pharmacopoeia*.”

2.1.2 Correlation of Codex standards with oils at different breakdown levels

The above diluted samples were analysed in triplicate for characteristics proposed by the Codex Stan 210 (1999) and include also fatty acid composition determined by gas liquid chromatography (Kock, 1988), Relative Density, Refractive Index, Iodine Value, Peroxide Value, Acid Value and Unsaponifiable Matter (all analyses according to prescribed protocol of AOAC, 1990). The data are presented in Figures 2 to 7.

2.1.3 Correlation of Acid Value and breakdown levels from samples obtained at different frying establishments

In total, 84 used oil samples, drawn by Environmental Health Officials (EHOs) from across S.A. were analysed as described above for their Acid Value and breakdown levels (i.e. PTG content) and the results obtained then compared.

2.2 Evaluation of unused oils and fats

In total 165 representative samples of refined unused oils, drawn by EHOs from shops and frying establishments across S.A., were subjected to PTG analysis. All analyses were performed in triplicate.

AV and PV were found to be the two parameters that correlated best with PTGs. These parameters were subjected to the multivariate polynomial search procedure of NCCS 2004 using an estimated model (Figure 8).

3. Results and discussion

3.1 Evaluation of current regulations

3.1.1 Theoretical breakdown levels vs. actual breakdown levels

According to Figure 1, the PTGs of the diluted samples ranged from as low as 1.26 % to as high as 23.01 %. A high correlation coefficient of $r^2 = 0.9939$ was obtained by comparing the theoretical and actual PTG values.

3.1.2 Relative % long-chain fatty acids vs. % breakdown levels

No significant change in the major fatty acid composition occurred when comparing the fatty acid composition of the diluted samples with the respective PTG values (Figure 2). The reason for this cannot be explained at present. It is clear from these results that total long-chain fatty acid values can not be used to monitor breakdown levels of used oils and fats.

3.1.3 Codex standards

3.1.3.1 Relative Density (Figure 3):

In total 17 of the dilutions showed in Figure 1 were analysed for Relative Density (RD). All experiments were performed at least in triplicate. Although a high correlation was

obtained when comparing the RDs of the oils with the % PTG values, the fluctuation (variation) in RD results when PTGs were between 1 % - 4 % were such that RDs can not be used as an accurate tool to determine breakdown levels and therefore mixing practices at these low concentrations.

3.1.3.2 Refractive Index (Figure 4):

A low correlation coefficient was found in the range tested when comparing Refractive Index (RI) and % PTGs. RI values can therefore not be used to determine breakdown products and mixtures accurately especially at low concentrations.

3.1.3.3 Iodine Value (Figure 5):

A poor correlation coefficient was obtained in the range tested when Iodine Value (IV) and % PTGs were compared. Consequently, IV values cannot be used to determine oil breakdown levels or mixtures accurately.

3.1.3.4 Peroxide Value (Figure 6):

A high correlation coefficient was obtained in the range tested when comparing Peroxide Value (PV) with % PTGs. This method however is prone to large variation between individual tests especially between laboratories due to the instability of peroxides that are measured and it is advisable not to use this in a routine method for law enforcement.

3.1.3.5 Acid Value - by titration (Figure 7):

Encouraging results were obtained when comparing Acid Value (AV) and PTG breakdown values. According to the Codex standards (Codex STAN 210, 1999), new

unused oils should not contain an Acid Value of more than 0.6 mg KOH/g Oil. Our results show a high reproducibility for AVs as well as a high correlation ($r^2 = 0.9664$) between the AVs and PTG values.

From these results, oils with an Acid Value of about 0.6 mg KOH/g oil should have a corresponding PTG value of approx. 4.7 %. From a practical point of view it therefore seems possible that the Acid Value according to Codex limits should be able to pick up mixed oils and fats at a somewhat higher PTG value as determined for the standard baseline limit i.e. 3 % (Oilseeds Advisory Committee Report, 2000). If this small deviation is acceptable, then the Acid Value, which is already included in the present legislation in its reference to the Codex, will be sufficient to detect mixed oils. Implications of these results are that any used oil with a PTG value of > 4.7 % may not be sold by any establishment or collectors for human consumption as it will not comply any more with the stipulations of reg. 2(4) as quoted in the footnote on pg 42. However, these values should always be confirmed with AV tests before law enforcement is considered.

3.1.3.6 Unsaponifiable Matter

No correlation was found in the range tested when comparing Unsaponifiable Matter and % PTGs (results not shown). This may be ascribed to the addition of the highly oxidised oil to unused oil thereby probably causing polymers and other unsaponifiable substances to form. Unsaponifiable Matter can therefore not be used to accurately monitor PTG breakdown levels in used or mixed oils and fats.

3.1.4 Correlation of Acid Value and PTG breakdown values from samples obtained at different frying establishments

It is important to realize that the high correlation obtained between Acid Value and % PTG was found under strictly regulated conditions i.e. where the same overused oil was diluted with the same unused oil to obtain a series of sequentially diluted samples that were tested for their Acid Value and PTG breakdown content. These experiments were subsequently expanded to include used oils from the different frying establishments in S.A.

It was found that 61 of the 84 samples had PTG values of equal to or greater than 3 %. Of these, 93.4 % had an Acid Value of greater than 0.6 mg KOH/g oil which renders the PTG technique usable to estimate the Acid Value when above the limit set by the Codex. Our surveys (i.e. testing the stability of frying oils for edible oil manufacturers at frying establishments) have shown that only one frying session will render an unused oil to have a PTG value of above 3 % and therefore also most probably an AV of close to or above 0.6 mg KOH/g of oil.

3.2 Do mixing practices still occur?

Representative samples (165) of refined, unused oils available in S.A. have been drawn by EHOs from shops and frying establishments across S.A. and PTG analysis was performed on each sample. All samples except one (i.e. 3.5 % PTGs), contained PTG breakdown levels of equal to and below 3 % (range: 0.1 - 3.0 %). According to these results and comparison with our previous baseline study (Oilseeds Advisory Committee

Report, 2000) we are glad to report that in most cases no mixing practices (i.e. used with unused oils) have been noticed as was reported previously (Kock et al., 1999). This may be ascribed to the elaborate awareness campaigns launched by us through SAFOI (S.A. Fryer Oil Initiative) as well as successful campaigns by the Health Department.

4. Conclusions

1. The present legislation* is adequate and prohibits any frying establishment to sell or distribute used oils and fats for re-use in the preparation of food – EVEN AFTER ONLY ONE USE!
2. The AV and PTG analyses methods are sensitive enough to uncover mixing practices of unused oils with low concentrations of used oils.
3. Mixing practices as determined in unused oil samples in the past, have declined dramatically since 2000, and can be ascribed to the awareness campaigns of the SAFOI network and Health Department.

* Section 2(1)a(iii) of Act no. 54 of 1972 prohibits the selling of a foodstuff.....
“(iii) which does not comply with any standard of composition, strength, purity or quality prescribed by regulation....” Regulation 2(4) of G.N. R 1316 of 1996 under the Act stipulates that: “The standards for the purity and composition of edible fats and oils shall be (if any) those laid down in the latest edition of the *Codex Alimentarius Standards for Fats and Oils* or the *British Pharmacopoeia*. Cooking oil does not comply anymore with the Codex standards even after being used only once. As the PTGs and total polar compounds are obviously still within the limits as prescribed in the regulations it can legally still be used by the particular frying establishment for the preparation of food. Such oil may however not be sold for human consumption by anyone!

5. Acknowledgements

The authors would like to thank the Oilseeds Advisory Committee for their support in conducting this study.

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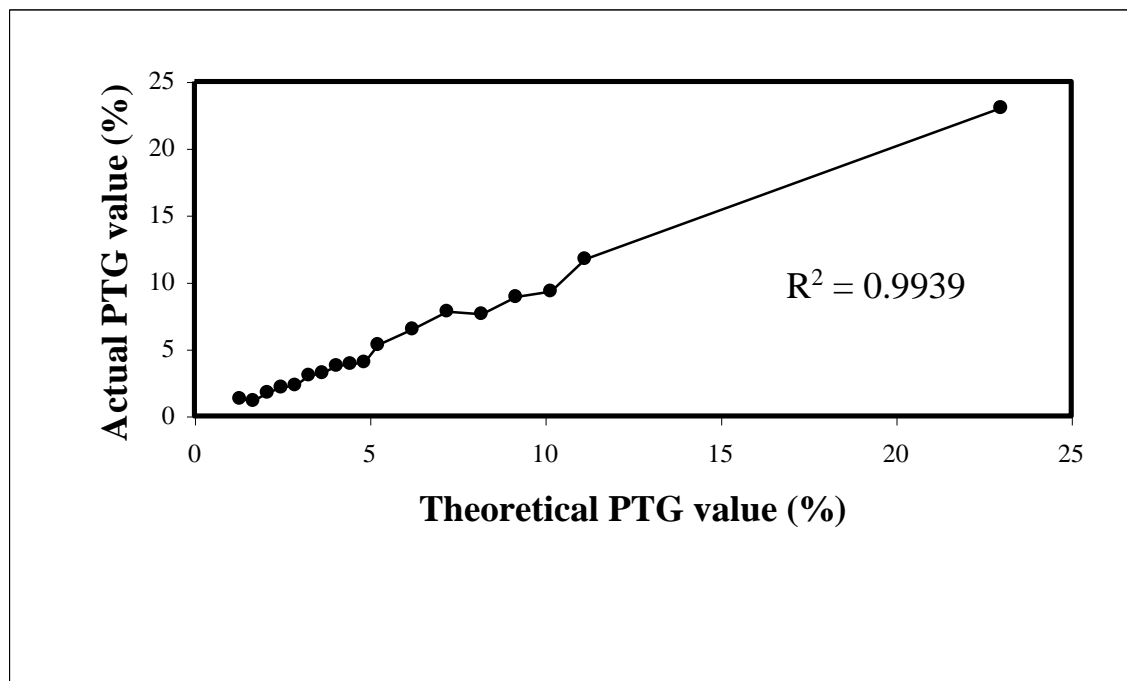


Figure 1. Scatterplot of theoretical % PTG vs actual % PTG (S.E. < 5%).

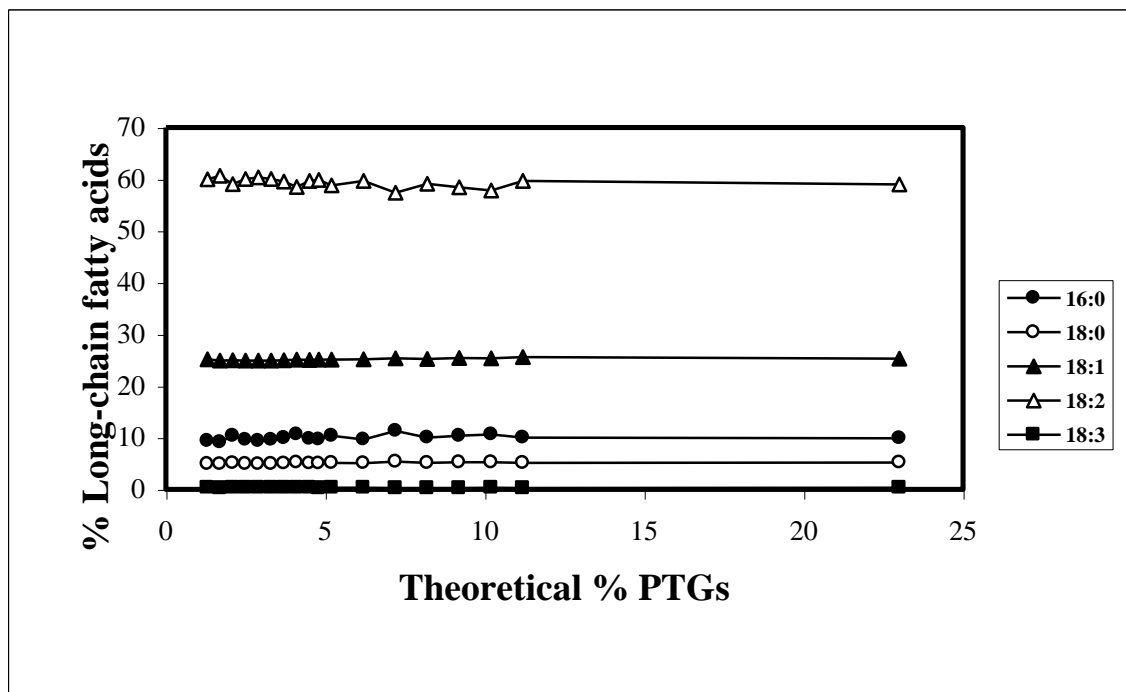


Figure 2. Relative % long-chain fatty acids vs theoretical % PTGs (S.E. < 5%).

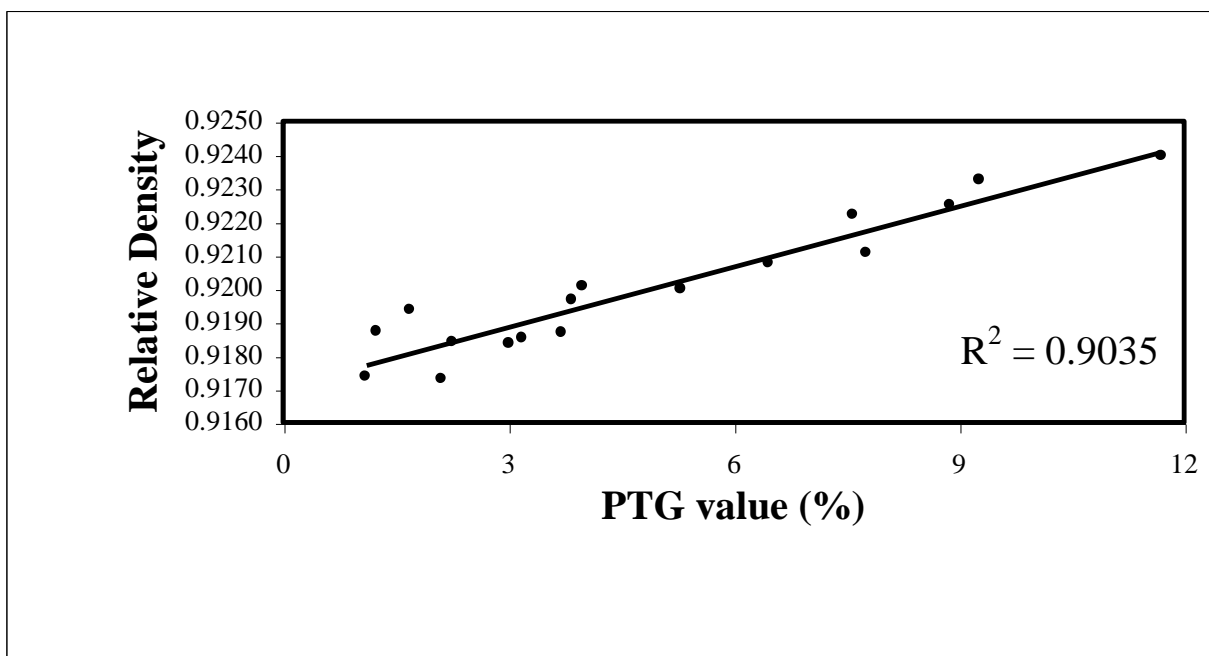


Figure 3. Scatterplot of theoretical % PTG vs Relative Density (S.E. < 5%).

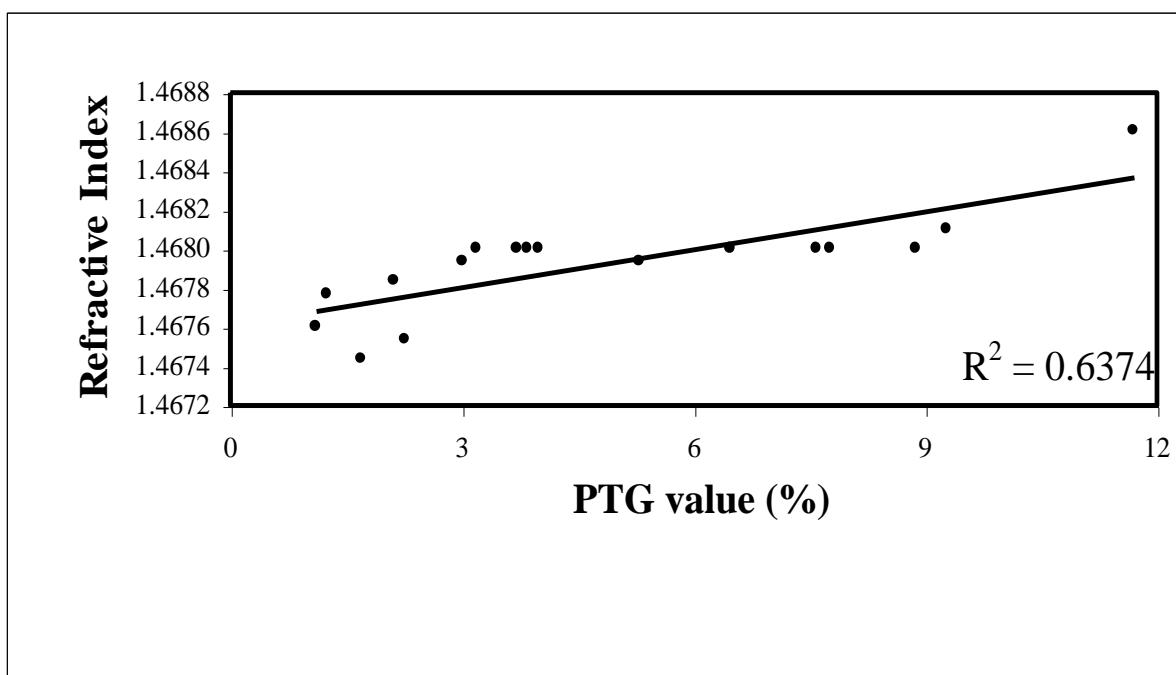


Figure 4. Scatterplot of theoretical % PTG vs Refractive Index (S.E. < 5%).

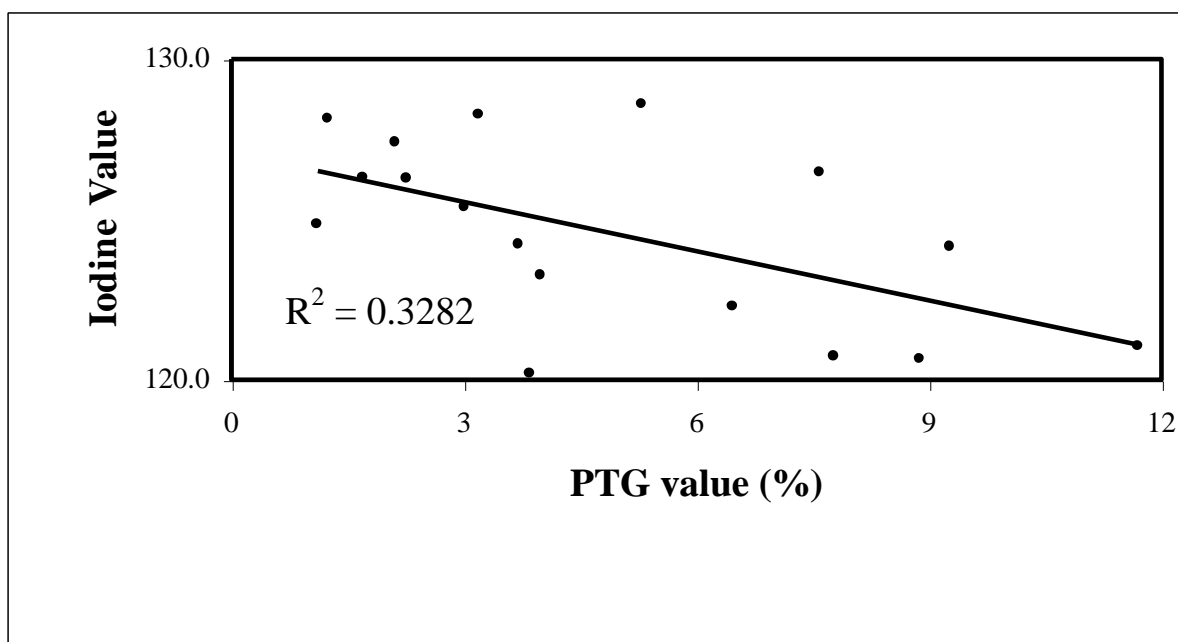


Figure 5. Scatterplot of theoretical % PTG vs Iodine Value (S.E. < 5%).

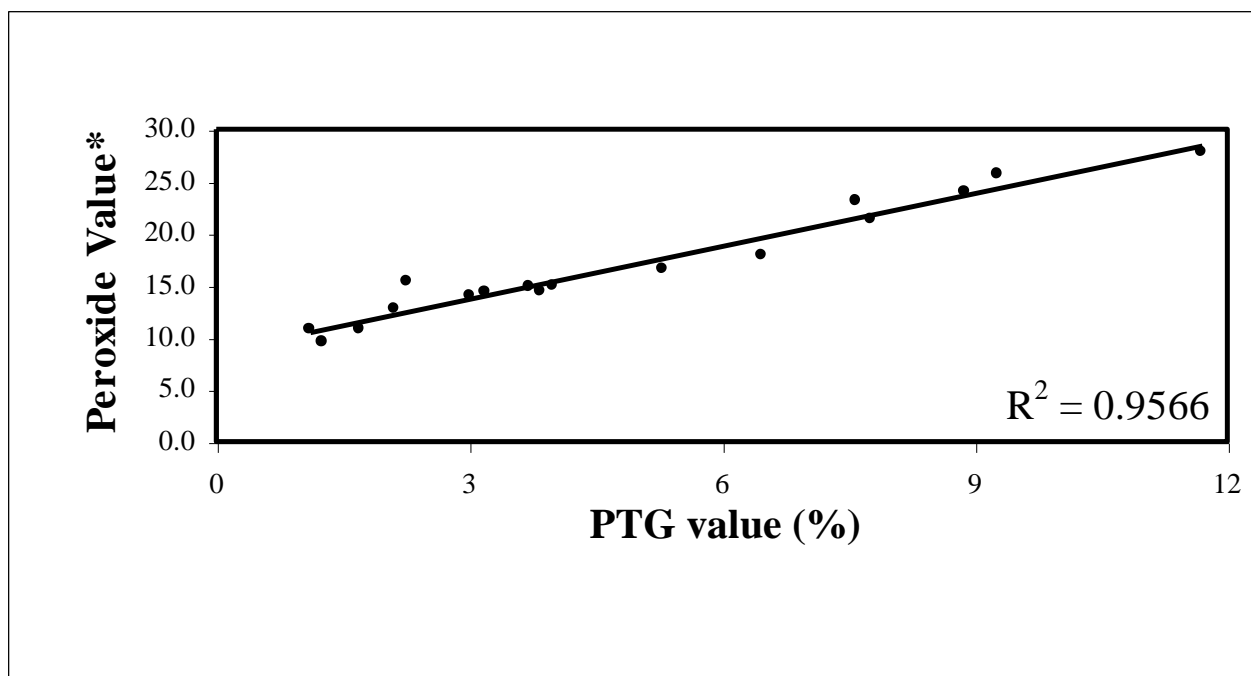


Figure 6. Scatterplot of theoretical % PTG vs Peroxide Value (S.E. < 5%). * = milliequivalents of active oxygen/kg oil.

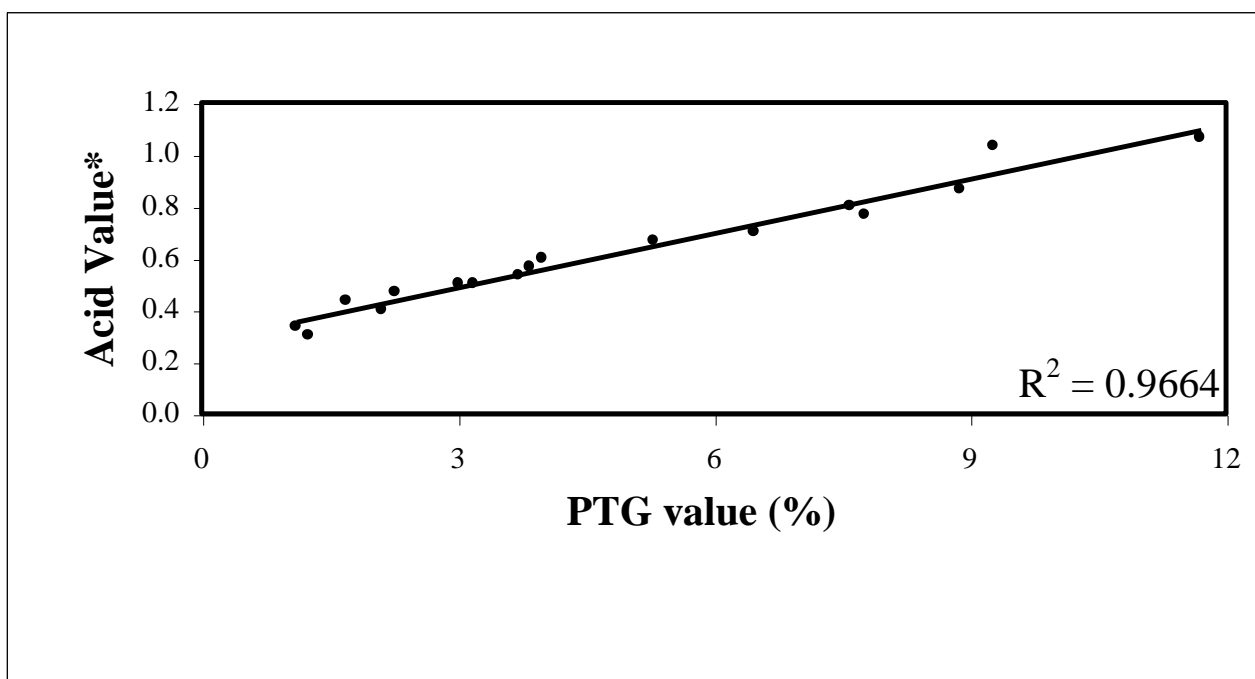


Figure 7. Scatterplot of theoretical % PTG vs Acid Value (S.E. < 5%). * = mg KOH/g oil.

The best transformation (In terms of R^2 value) was subjected to the multivariate polynomial fit procedure of NCCS 2004. The model used is indicated below. The high R^2 value ($R^2 = 0.992268$) gives an indication of the accuracy of the model.

Estimated Model

$$\begin{aligned} \text{PTG} = & ((9.559225)-(18.14927)*(\text{SQRT}(P))+(13.03574)*(\text{SQRT}(P))^2- \\ & (4.292918)*(\text{SQRT}(P))^3+(0.6073075)*(\text{SQRT}(P))^4+(28.4128)*A- \\ & (30.76943)*(\text{SQRT}(P))*A+(6.243964)*(\text{SQRT}(P))^2*A+(2.339801)*(\text{SQRT}(P)) \\ & ^3*A-(0.691321)*(\text{SQRT}(P))^4*A- \\ & (19.43672)*A^2+(71.77533)*(\text{SQRT}(P))*A^2- \\ & (49.68064)*(\text{SQRT}(P))^2*A^2+(9.517023)*(\text{SQRT}(P))^3*A^2- \\ & (157.1856)*A^3+(165.709)*(\text{SQRT}(P)) \\ & *A^3-(40.71752)*(\text{SQRT}(P))^2*A^3- \\ & (156.8196)*A^4+(70.48748)*(\text{SQRT}(P))*A^4) / (1- \\ & (2.521219)*(\text{SQRT}(P))+(2.227808)*(\text{SQRT}(P))^2- \\ & (0.936554)*(\text{SQRT}(P))^3+(0.1965863)*(\text{SQRT}(P))^4+(6.624337)*A- \\ & (9.664564)*(\text{SQRT}(P))*A+(5.353977)*(\text{SQRT}(P))^2*A- \\ & (1.407968)*(\text{SQRT}(P))^3*A+(0.1552045)*(\text{SQRT}(P))^4*A+(6.103373)*A^2- \\ & (5.525272)*(\text{SQRT}(P))*A^2+(2.179912)*(\text{SQRT}(P))^2*A^2- \\ & (0.4098482)*(\text{SQRT}(P))^3*A^2- \\ & (3.560498)*A^3+(2.029353)*(\text{SQRT}(P))*A^3+(0.1690818)*(\text{SQRT}(P))^2*A^3- \\ & (4.15786)*A^4+(0.3799154)*(\text{SQRT}(P))*A^4) \end{aligned}$$

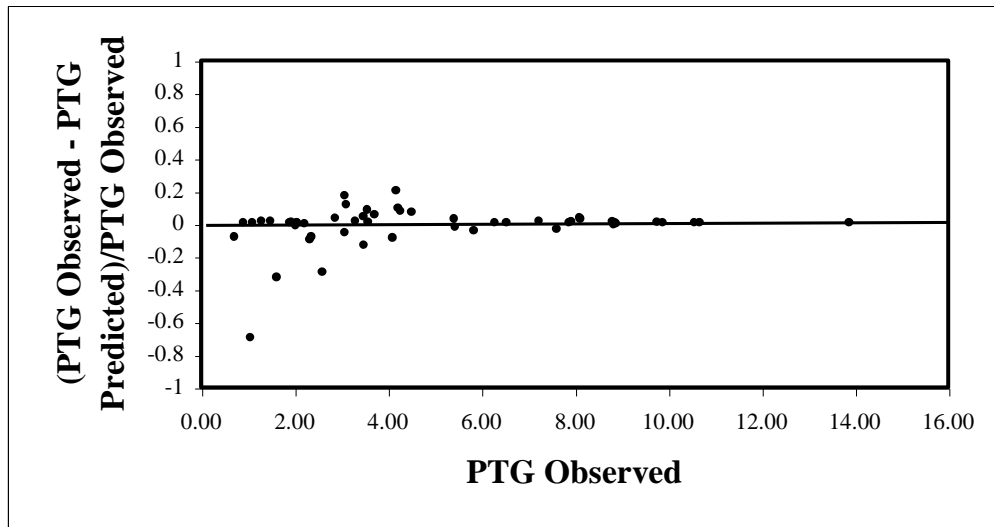


Figure 8. Scatterplot of actual % PTG vs actual % PTG-theoretical % PTG/actual % PTG (S.E. < 5%).

Chapter 3

The influence of acetate and polymerised triglyceride content **on edible oil utilisation by *Mucor***

Parts of this chapter have been accepted for publication as:

Acetate-enhanced polymerised triglyceride utilisation by *Mucor circinelloides*

Joseph *et al.* 2004

World Journal of Microbiology and Biotechnology

Abstract

When *Mucor circinelloides f. circinelloides* CBS 108.16 was cultivated on a mixed substrate of sunflower oil (30 g/l) containing 1 %, 5 %, 10 %, 15 % and 45 % (w/w) polymerised triglycerides (PTGs) and sodium acetate (10 g/l) as carbon sources, most of the oil was utilized after seven days of growth. Marked increases in biomass production as well as in the lipid content of the fungal cells were also noted. Strikingly, these sunflower oils were transformed to fungal oils containing gamma-linolenic acid (GLA) and substantial amounts of linoleic acid (18:2) - also characteristic of evening primrose oil. This phenomenon was however not observed in the medium containing only oils with variable amounts of PTGs (40 g/l) and no acetate. In the presence of acetate, there was an increase in the pH of the medium from pH 5.8 to about neutrality as well as consumption of PTGs from the growth medium whereas in the absence of acetate there was a decrease in the medium pH from pH 5.8 to around pH 3.0 while the relative amounts of PTGs kept increasing in the extracellular lipids. The highest amounts of fungal oils were produced by *M. circinelloides* using the 5 % and 10 % (w/w) PTG oils. This study suggests the production of Evening Primrose Oil equivalents (EPOeq) from sunflower oil using *Mucor*.

1. Introduction

More than 100 000 tonnes of frying oil waste are produced from the estimated 54 000 frying establishments in South Africa per annum. Some of these oils contain breakdown products such as polymerised triglycerides (PTGs), which are harmful to human health (Anelich *et al.* 2001), when produced in concentrations greater than 16 % (w/w). It was

found that PTG values of up to approximately 40 % of total oil content could accumulate during repeated oil use in frying practices (Pelesane *et al.* 2001; Kock *et al.* 2002). According to South African food regulations, oil with a PTG content of equal to or above 16 % (w/w) is considered unsafe for use (Anelich *et al.* 2001). Studies so far by other researchers such as Jeffery *et al.* (1997) and Pelesane *et al.* (2001) have shown that fresh and overused sunflower oils (containing 44 % w/w PTGs) can be transformed by fungi in the presence of acetate to high value lipids such as gamma-linolenic acid (GLA). In the presence of acetate, a marked increase in fungal mass and GLA content was observed when compared to growth conditions using sunflower oil as the sole carbon source. This poses the possibility of developing a biotechnological route where fungi such as *Mucor* can be used to transform safe used oil waste containing low % PTGs within regulatory limits i.e. less than 16 % w/w, to safe high value lipids containing GLA.

Plant oil containing GLA, such as Evening Primrose Oil (EPO), is being used in increasing amounts in nutritional and pharmaceutical preparations, as there are claims that it may alleviate certain chronic disease states such as cancer, heart disease and eczema (Horrobin 1990; Christie 1999). As a result of this, substantial amounts of high value EPO is used worldwide i.e. approximately 1500 tonnes per annum at about US \$ 60 per kg (Lapinskas 2000). The replacement of such high value plant lipids by fungal lipids has been targeted by researchers and industry as an important field of biotechnological research (Ratledge 1993). According to Ratledge (1993), there are potentially three markets, which single cell oil (SCO) products may influence. These

include cocoa butter, gamma-linolenic acid and some polyunsaturated fatty acids (PUFAs) such as eicosapentaenoic acid and arachidonic acid.

Consequently, the aim of this study became to investigate the transformation of used oils with different PTG values i.e. 1 %, 5 %, 10 % and 15 % (w/w) within the regulatory limits and oil with a very high PTG content i.e. 45 % (w/w) to high value oils similar to that of Evening Primrose Oil.

2. Materials and methods

2.1 Preparation of edible oils with varying amounts of PTGs

Unused sunflower oil (PTG = 1.0 % w/w) was heated at a temperature of 200 °C with continuous stirring and aeration to simulate the frying process thereby forming PTGs under more controlled conditions using a Millipore vacuum pump XF54 230 50 until the desired PTG levels of 1 %, 5 %, 10 %, 15 % and 45 % (w/w) were obtained. High Performance Liquid Chromatography analysis using a Hewlett Packard HP 1047A chromatograph equipped with a RD 1 detector was carried out to determine the PTG levels.

2.2 Microorganisms and cultivation

Mucor circinelloides f. circinelloides CBS 108.16 was transferred from 4-day-old YM (yeast-malt-agar) slants (incubated at 25°C) to 100 ml sterile growth medium (pH 5.8) contained in 1 l conical flasks. The medium consisted of the following in g/l: oils with

variable PTG amounts, 30; sodium acetate, 10; yeast extract, 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; K_2HPO_4 , 10.0; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05; NH_4Cl , 1.28. Trace elements were added to a final concentration (g/l) as follows: $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.035; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.007; $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.011; $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.001; $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.002; $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.0013; H_3BO_3 , 0.002; KI , 0.00035; $\text{Al}_2(\text{SO}_4)_3$, 0.0005. Cultures, incubated at 25 °C for seven days while being shaken at 160 rev/min, were harvested for analysis by filtration (Whatman No.1) at different intervals as indicated in Figure 1. All experiments were performed at least in duplicate. After harvesting, cells were washed extensively and then immediately frozen and freeze-dried. As a control experiment, the same medium as above was used with the exception that oils with variable amounts of PTGs (30 g/l) and sodium acetate (10 g/l) were replaced by sunflower oil (40 g/l; PTGs = 1 %, 5 %, 10 %, 15 % and 45 % w/w) as the sole carbon source.

2.3 Lipid extraction

The extraction and fractionation of lipids were performed as described by Kock *et al.* (1997). In short, extracellular lipids present in the corresponding supernatant (pH < 4) from each flask were immediately extracted with hexane until no lipids were detected. Intracellular lipids were extracted from freeze-dried cells using chloroform/methanol (2:1 v/v) as described by Folch *et al.* (1957), followed by two washes with distilled water and final evaporation of the organic phase under vacuum. Subsequently, the lipid material was then dissolved in diethyl ether and transferred to pre-weighed vials. Prior to determination of lipid weights, samples were dried to constant weight in a vacuum oven over P_2O_5 at 55 °C.

2.4 Polymerised triglyceride analysis

Both intracellular and extracellular lipids were dissolved in tetrahydrofuran (THF) and polymers, which included products formed by carbon to carbon and/or carbon to oxygen linkages between mono-, di and triglyceride bound fatty acids, as well as free fatty acids, to produce dimeric or higher polymeric compounds, were determined by gel permeation chromatography as described by Beljaars (1993).

2.5 Fractionation of extracted lipids

The extracted lipids were dissolved in chloroform and then applied to a column (140 mm x 20 mm) of silicic acid, activated by heating overnight. The neutral (NL), glyco (GL) and phospho (PL) lipid fractions were obtained by applying organic solvents of different polarities as described by Kendrick & Ratledge (1996). Final solvent removal and storage were as for whole lipid extracts.

2.6 Fatty acid analyses

Lipids were dissolved in chloroform and methylated by the addition of trimethyl sulphonium hydroxide (TMSOH) as described by Butte (1983). The fatty acid methyl esters were analysed by gas chromatography, with a flame ionisation detector and a Supelcowax 10 capillary column (30 m x 0.75 mm). The initial column temperature of 145 °C was increased by 3 °C/min to 225 °C and, following a 10 minute isothermal period, was increased to 240 °C at the same rate. The inlet and detector temperatures

were 170 °C and 250 °C respectively. Nitrogen was used as a carrier gas at 5 ml/min. Peaks were identified by reference to authentic standards.

2.7 Acetic acid analyses

Residual acetic acid present in the supernatants of all experiments was determined by using a Hewlett Packard gas chromatograph equipped with a flame ionisation detector and a 30 m x 0.53 mm Nukol capillary column. The oven temperature of 120 °C was increased at a rate of 6 °C/min to 165 °C. The inlet and detector temperatures were 135 °C and 250 °C, respectively. The carrier gas was nitrogen at a flow rate of 5 ml/min. The samples and acetic acid standard (1 ml) were acidified with 0.3 ml of a 25 % formic acid solution prior to analysis.

2.8 Chemicals

All organic chemicals and solvents used were of analytical reagent grade and obtained from major retailers. Silicic acid (100 mesh) was from Aldrich. Fatty acid standards were from Sigma.

3. Results and discussion

In the presence of a substrate mixture of sunflower oil with variable amounts of PTGs (5 %, 10 %, 15 % and 45 % w/w) and sodium acetate, *Mucor circinelloides f. circinelloides* grew significantly better than when grown on sunflower oil containing various PTG concentrations as the sole carbon source (Figure 1). Maximum biomass was produced

within 96 h in the presence of acetate. The highest biomass was obtained on 5 % and 10 % (w/w) PTG containing oils with acetate. Here 23.2 g dry weight/l and 22.6 g dry weight/l (Figure 1) containing as much as 71.3 % and 75.2 % (w/dry weight) crude lipids (Figure 2) were found respectively. In all experiments, the crude lipids contained more than 90 % neutral lipids (results not shown).

In general, a reduction in intracellular lipid content was experienced following growth from 96 h to 120 h (Figure 2). This could be due to a switchover of the energy metabolism to the utilization of intracellular lipids in the presence of acetate, since the sunflower oil in the medium became exhausted after 96 h (Figure 3). The biggest decrease in the intracellular lipid content from 96 h to 120 h in the presence of acetate was observed with the 45 % (w/w) PTG oil. This decrease in the intracellular lipid content may be ascribed to the presence of lower amounts of usable oil present in the medium with increasing amounts of probably unusable PTGs (Figures 3 & 6).

In the presence of sodium acetate, significantly larger amounts of oil were utilized than in its absence (Figure 3). In the presence of acetate, oils containing 5 %, 10 % and 15 % (w/w) PTGs were extensively utilized (97 %; 98 %; 94 % w/w) after 168 h respectively, while the oil containing 45 % PTGs was utilized to a lesser extent (83 % w/w) after 168 h (Figure 3). This may be ascribed to the presence of increased amounts of polymers present in the oils. In the absence of acetate, oils containing 5 %, 10 %, 15 % and 45 % (w/w), PTGs were less utilized i.e. 50.0 %; 37.0 %; 24.8 %; 22.8 % (w/w) respectively

after 168 h (Figure 3). These findings are in accordance with literature (Jeffery *et al.* 1999; Pelesane *et al.* 2001) who observed the same for 44 % (w/w) PTG oils.

Acetic acid was almost completely exhausted after 72 h (Figure 4), with an increase in the pH of the medium close to neutrality, whereas in the absence of acetate, the medium pH decreased to about pH 3.0 in all cases (Figure 5). Complete emulsification of sunflower oil with variable amounts of PTGs in the presence of acetate was observed within 20 h during the utilization of the combined substrate mixture, whereas in the absence of acetate, no emulsification was observed after 168 h of growth. In this study, used frying fat is regarded to be completely emulsified when all fat is fully dispersed i.e. no oil droplets were visible at the surface of the growth medium when motionless for 5 minutes. The reason for the acetate-supported emulsification remains to be clarified. Emulsification of free fatty acids produced by lipase activity at neutral pH may enhance this process (Verhagen *et al.* 1978). It is interesting to note that emulsification of the oils favours utilization for cell growth and production of fungal lipids.

Interesting patterns were observed in the extracellular and intracellular lipid PTG content when grown in the presence and absence of acetate (Figures 6 & 7). In general, with the exception of the 45 % (w/w) PTG oil, all the PTGs were utilized from the medium within 72 h of growth in the presence of acetate. In the presence of acetate and 45 % PTG oil, there was a gradual decrease in the amount of PTGs to approximately 12 % (w/w) in the medium after 168 h (Figure 6). Strikingly, in the absence of acetate, there was a gradual increase in the relative amounts of extracellular PTGs to approximately 25 % (w/w) when

cultivated in 5 %, 10 % and 15 % (w/w) PTG oils. Much less PTGs were utilized over the growth cycle when cultivated on the 45 % (w/w) PTG oil while no increase in the relative % PTGs was observed (Figure 6). The increase in the relative amounts of extracellular PTGs in the absence of acetate could be due to a preference towards the normal non-polymerised oil thus resulting in a relative increase in PTGs.

In general, as expected, no PTGs were present intracellularly at the start of growth (Figure 7). In the presence of acetate, with the exception of the 45 % (w/w) PTG oil, no PTGs were present intracellularly after 168 h. It can be assumed that the PTGs were utilized, since they disappeared from the growth medium and were absent in the intracellular lipids. With the 45 % (w/w) PTG oil, in the presence of acetate (Figure 7), the PTGs increased inside the cell over the growth cycle implying that they were taken up by the cell. In the absence of acetate, the PTGs kept increasing inside the cell to reach values of about 45 % (w/w) for (5 % and 15 % w/w PTG oils), after 168 h of growth. Interestingly the increase in PTGs with the 45 % (w/w) PTG oil in the absence of acetate was much lower, and reached approximately 15 % (w/w) of biomass after 168 h of growth (Figure 7).

In the presence of acetate, but not in its absence, *Mucor* apparently exhibited a preference for the utilization of unsaturated fatty acids (FAs) i.e. mono and polyunsaturated FAs (results not shown). This is similar to that found by Jeffery *et al.* (1999) and Pelesane *et al.* (2001).

Table 1 shows the effect of PTGs on the cell turnover after seven days of growth in the presence and absence of acetate. Generally, much better cell turnover i.e. oil utilization, biomass production, intracellular lipid content, evening primrose oil equivalent production, was obtained in the presence of acetate than its absence. As previously reported by other researchers (Jeffery *et al.* 1997; Pelesane *et al.* 2001) it was found that the simultaneous metabolism of acetate markedly improved the conversion of oils - also those containing variable amounts of PTGs (1 %; 5 %; 10 %; 15 % and 45 % w/w) respectively.

At the end of the seven day growth period, the highest amounts of EPO equivalent were produced by the 5 %, 10 % and 15 % (w/w) PTG containing oils respectively. This suggests that oils within the regulatory PTG limits are easier taken up and transformed compared to higher PTG (45 % w/w) containing oils. From the results obtained, it is clear that a higher content of PTGs does have an effect on cell turnover (Table 1). The fatty acid profiles obtained in the intracellular lipids for all PTG containing oils tested, were similar to that of EPO but had higher amounts of 18:1 and lower amounts of 18:2 and 18:3 fatty acids (Table 2). Perhaps with further optimisation studies, this could be improved.

4. Conclusions

1. The study has shown that increasing amounts of PTGs have negative effects on cell turnover in general. This is clearly seen with the 45 % (w/w) PTG containing oil where much less oil was utilized from the growth medium when compared to the other four PTG concentrations. Also, the reduction in biomass, intracellular lipid content and EPOeq production is confirmatory that a higher PTG content does affect cell turnover negatively. It can be concluded that oils within the regulatory limits i.e. containing less than 16 % (w/w) PTGs were best suited for transformation studies.

2. The fatty acid profiles obtained are similar to that of Evening Primrose Oil (EPO) however lesser amounts of the biologically important fatty acids i.e. 18:2 and 18:3 were obtained. More research will have to be conducted using optimisation studies to improve the yield of the fatty acids of fungal oils such that it is more comparable to that of EPO.

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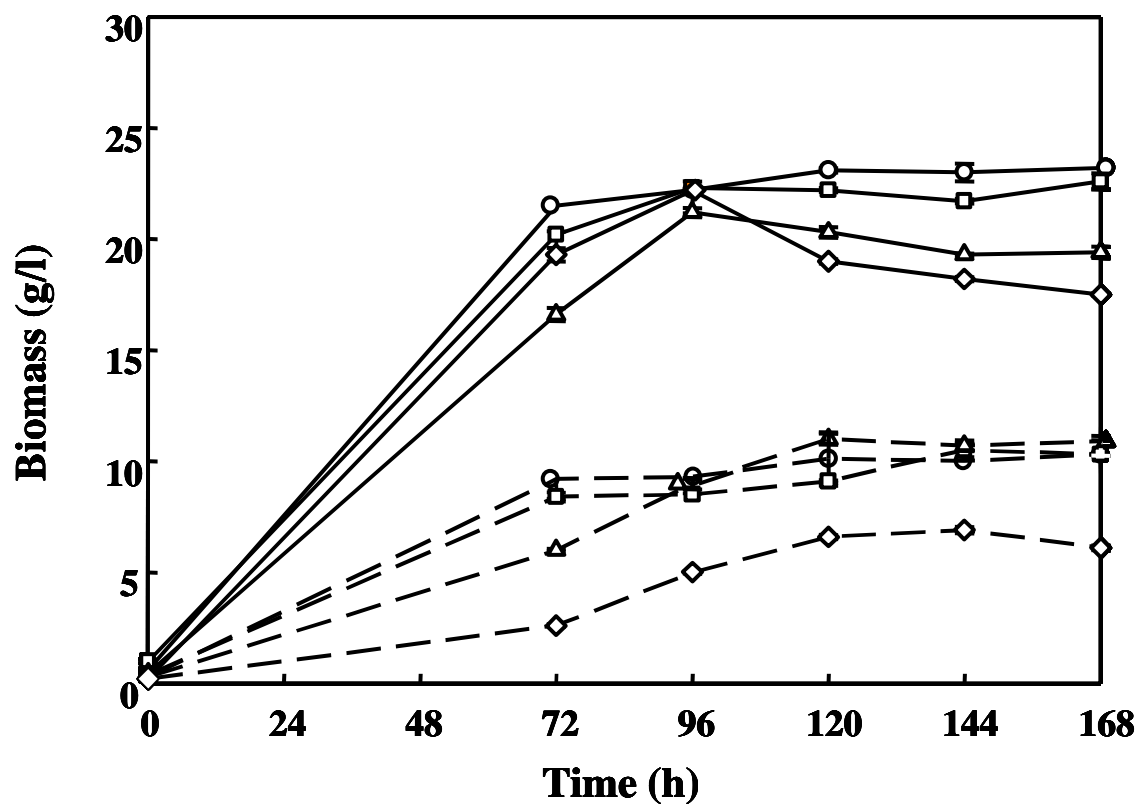


Figure 1. Biomass production by cultures grown in shake flasks for seven days at 25°C on used oils containing 5 % (○), 10 % (□), 15 % (△) and 45 % (◇) polymerised triglycerides in the presence (—) and absence (---) of acetate.

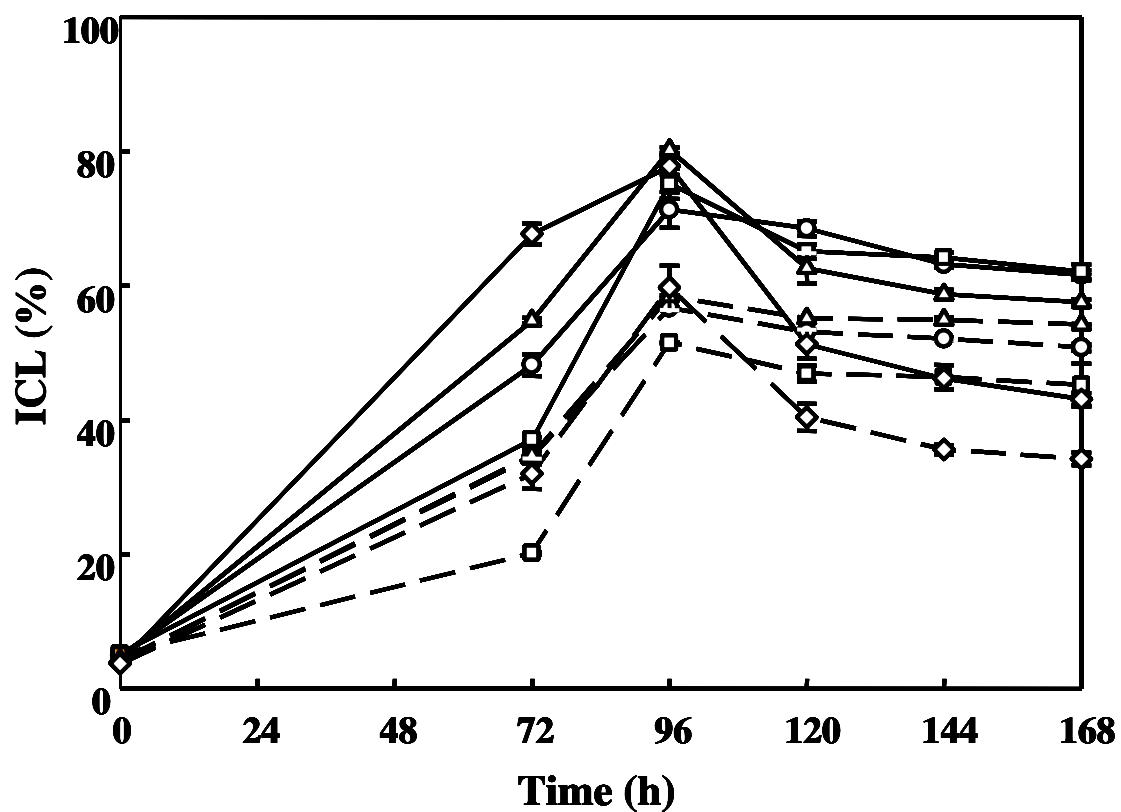


Figure 2. Intracellular lipid content of cultures grown in shake flasks for seven days at 25°C on used oils containing 5 % (○), 10 % (□), 15 % (△) and 45 % (◇) polymerised triglycerides in the presence (—) and absence (---) of acetate. ICL = Intracellular lipid content.

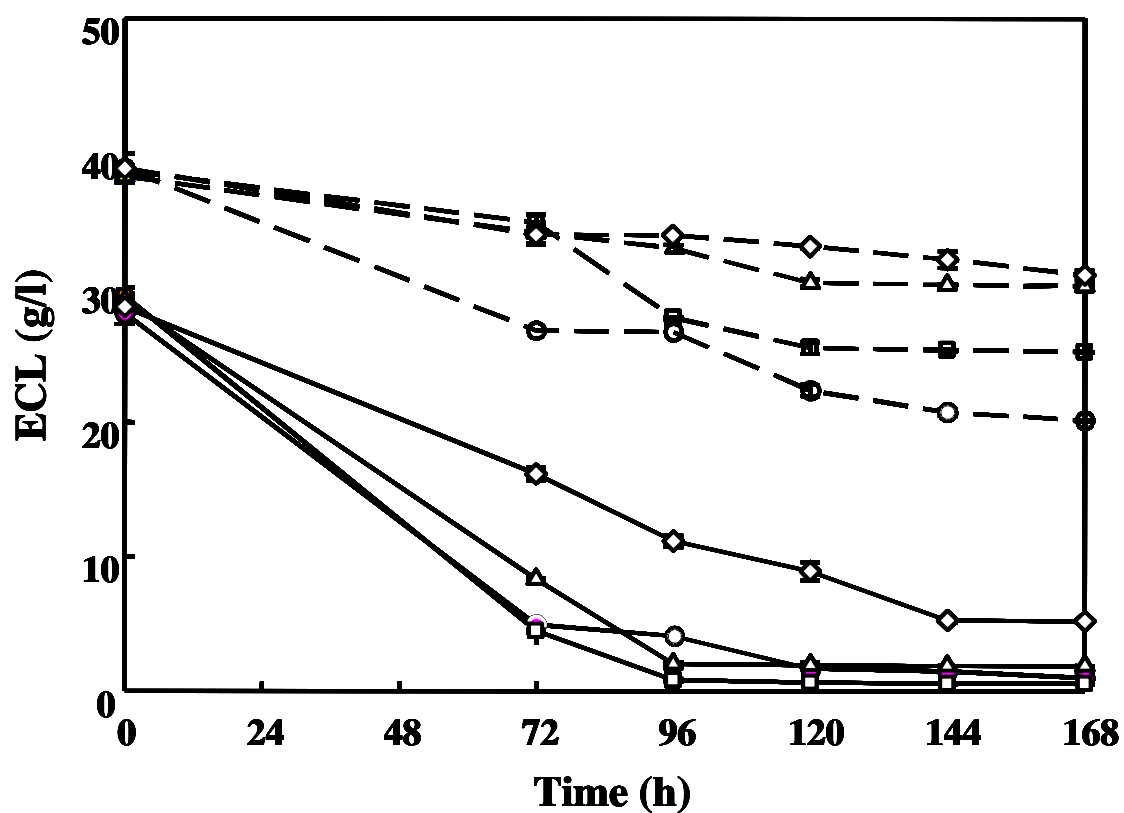


Figure 3. Oil utilisation by cultures grown in shake flasks for seven days at 25°C on used oils containing 5 % (○), 10 % (□), 15 % (△) and 45 % (◇) polymerised triglycerides in the presence (—) and absence (---) of acetate. ECL = Extracellular lipid content.

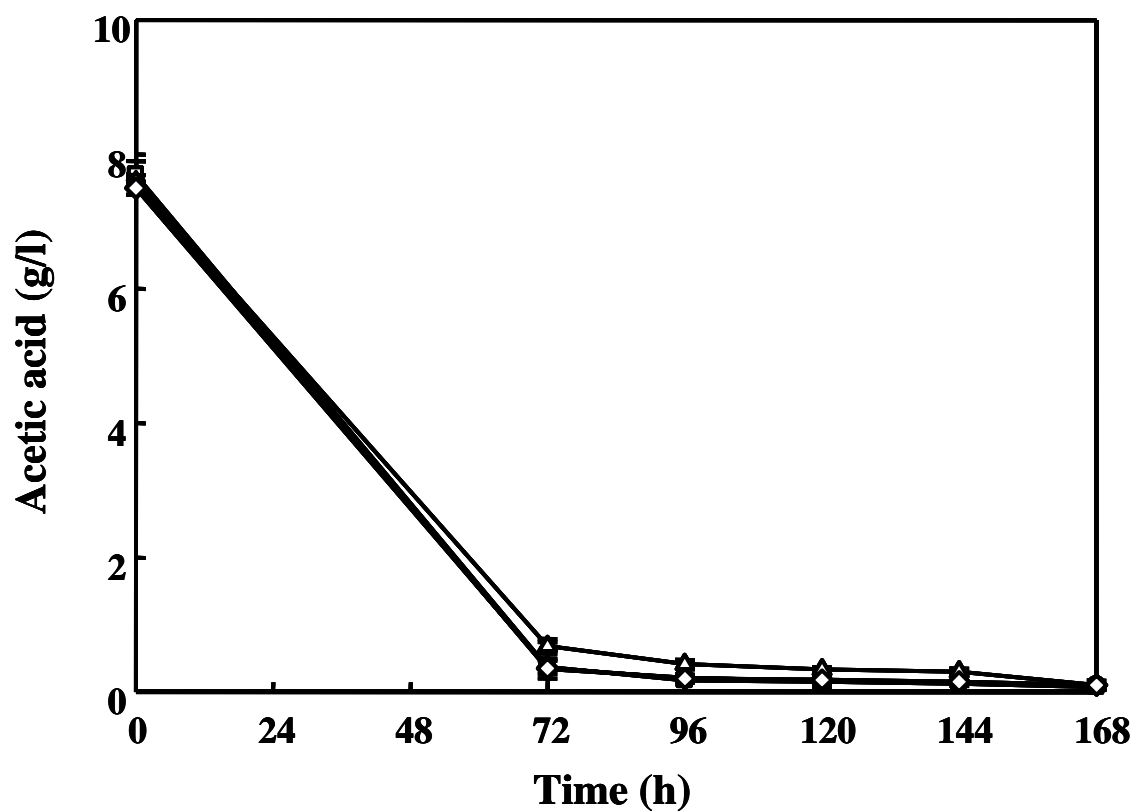


Figure 4. Changes in acetic acid content of cultures grown in shake flasks for seven days at 25°C on used oils containing 5 % (○), 10 % (□), 15 % (△) and 45 % (◇) polymerised triglycerides in the presence (—) of acetate.

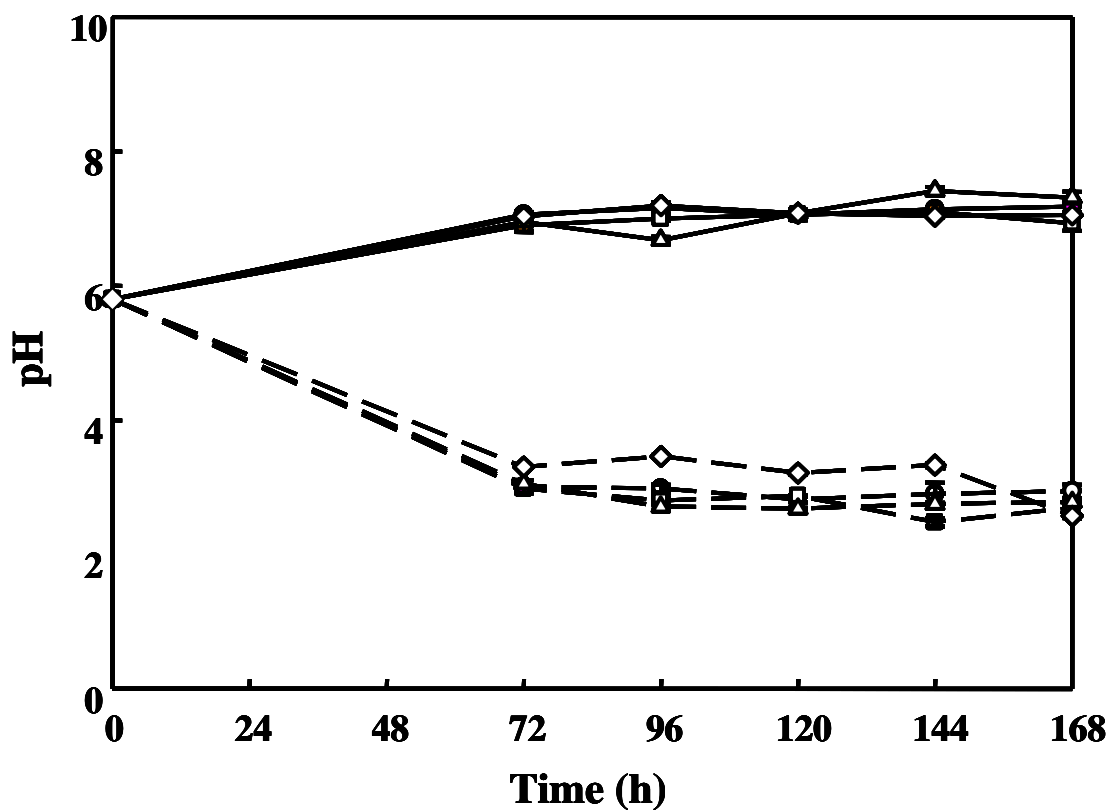


Figure 5. Changes in pH profiles of cultures grown in shake flasks for seven days at 25°C on used oils containing 5 % (○), 10 % (□), 15 % (△) and 45 % (◇) polymerised triglycerides in the presence (—) and absence (---) of acetate.

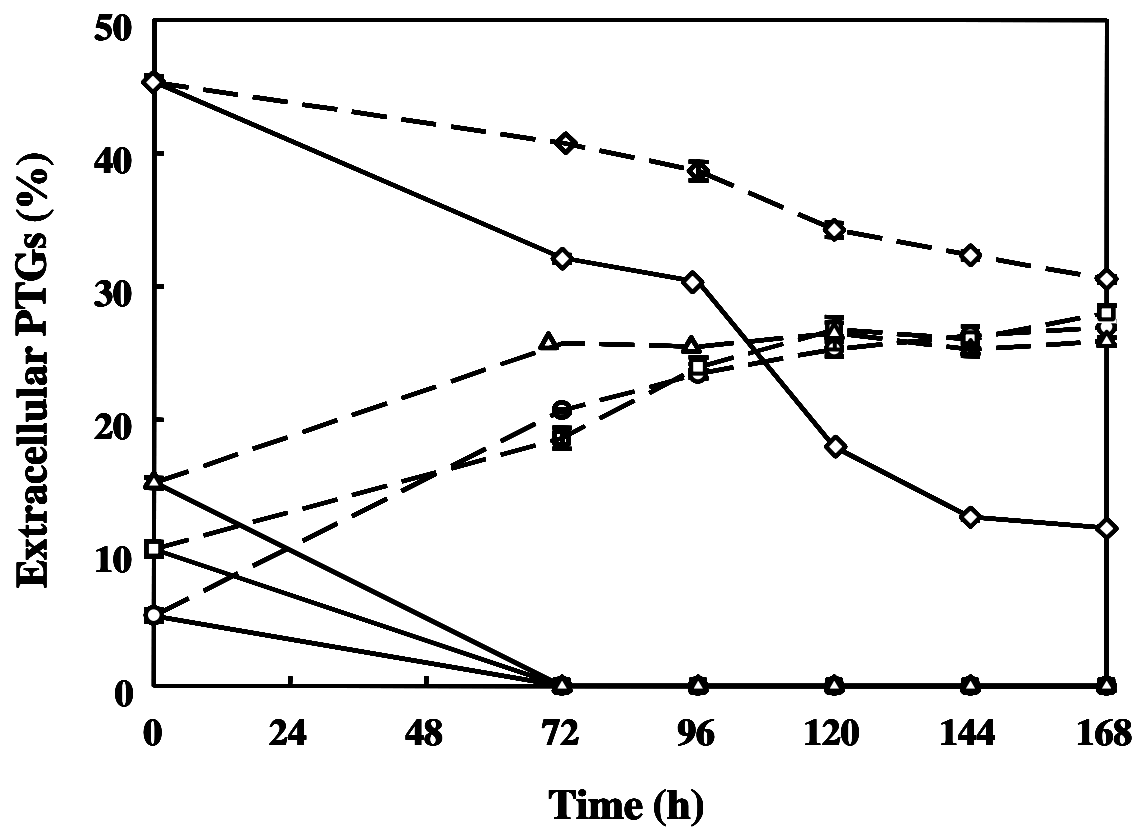


Figure 6. Extracellular polymerised triglyceride (PTG) content of cultures grown in shake flasks for seven days at 25°C on used oils containing 5 % (○), 10 % (□), 15 % (△) and 45 % (◇) polymerised triglycerides in the presence (—) and absence (---) of acetate.

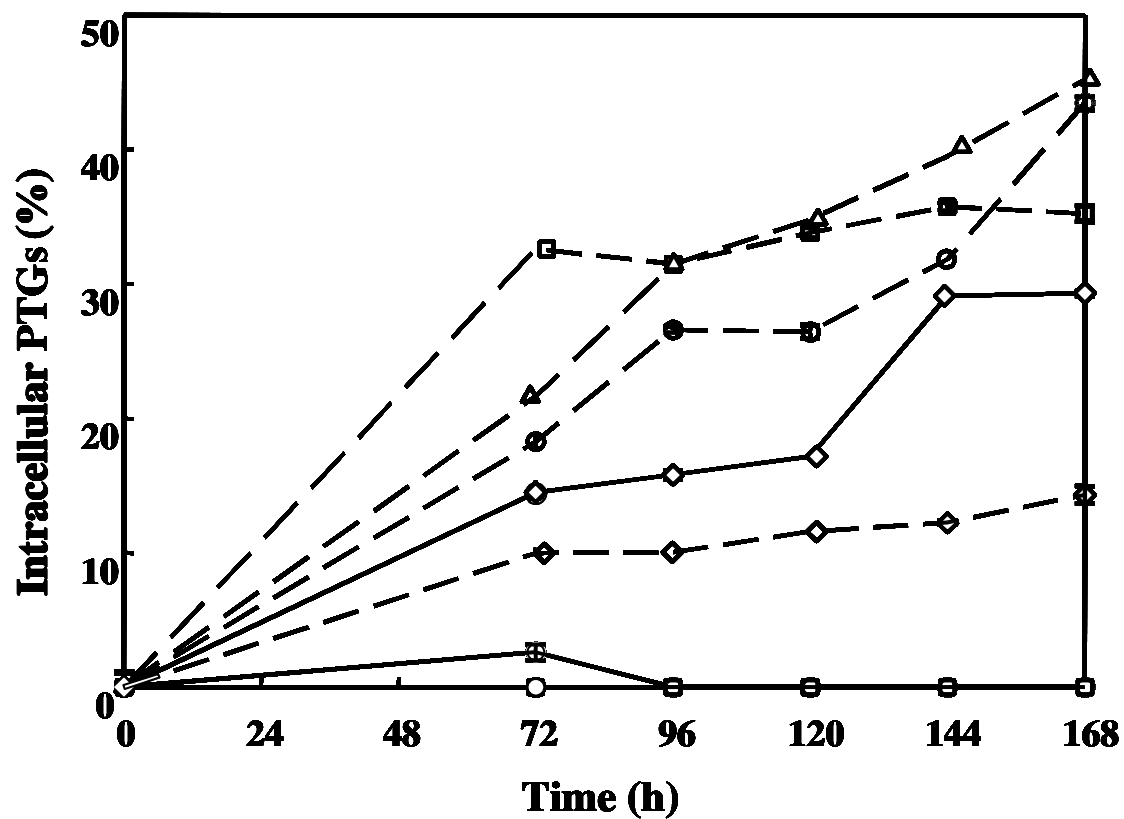


Figure 7. Intracellular polymerised triglyceride (PTG) content of cultures grown in shake flasks for seven days at 25°C on used oils containing 5 % (○), 10 % (□), 15 % (△) and 45 % (◇) polymerised triglycerides in the presence (—) and absence (---) of acetate.

Table 1. Effect of polymerised triglycerides (PTGs) on cell turnover and EPOeq production after seven days of growth in polymer containing oils in the presence (+) and absence (-) of acetate.

		1 %	5 %	10 %	15 %	45 %
pH	+	7.2	7.2	6.9	7.3	7.1
	-	2.7	2.9	2.7	2.8	2.6
AA (g/l)	+	0.1	0.1	0.1	0.1	0.1
	-	-	-	-	-	-
ECL (g/l)	+	2.4	0.9	0.5	1.8	5.2
	-	30.9	20.1	25.2	30.1	30.9
BM (g/l)	+	23.6	23.2	22.6	19.4	17.5
	-	3.6	10.3	10.3	10.9	6.1
ICL (%)	+	43.8	61.8	62.1	57.5	43.1
	-	38.0	50.8	45.1	54.2	34.2
EPOeq (g/l)	+	10.3	14.3	14.0	11.1	7.5
	-	1.4	5.2	4.6	5.9	2.1

AA: Acetic Acid; ECL: Extracellular lipid content; BM: Biomass; ILC: Intracellular lipid content; EPOeq: Evening primrose oil equivalent; PTGs: Polymerised triglycerides; + = Presence of acetate; - = Absence of acetate (% SE = < 5%).

Table 2. Percentage fatty acid content of Evening Primrose Oil (EPO) and EPOeq (*Mucor*) after seven days of growth.

PTGs	OILS	FATTY ACIDS (%)						EPOeq (g/l)
		16:0	16:1	18:0	18:1	18:2	18:3	
	EPO	6.8	0.8	3.2	8.6	68.5	8.6	
	SO	6.6	0.1	0.01	28.8	58.5	-	
1 %		6.4	0.6	2.1	21.0	58.8	3.3	10.3
5 %		8.9	0.9	1.3	29.2	50.4	4.0	14.3
10 %		10.7	1.0	3.4	24.2	44.6	3.6	14.0
15 %		7.8	0.1	5.9	24.2	54.3	3.3	11.1
45 %		7.2	0.3	3.6	26.5	48.4	3.1	7.5

EPO: Evening primrose oil; EPOeq: Evening primrose oil equivalent; PTGs: Polymerised triglycerides; SO: Sunflower oil (% SE = < 5%).

Chapter 4

Stereospecific analysis of Evening Primrose Oil Equivalents

(EPOeq) using quantitative Nuclear Magnetic Resonance

(NMR) and Gas Chromatography (GC)

1. Introduction

Having established the effect of varying amounts of polymerised triglycerides (PTGs) on Evening Primrose Oil equivalents (EPOeq) production in Chapter 3, the investigation is continued to determine the stereospecific position of fatty acids of EPOeq. This is since the positional distribution of fatty acids on the glycerol moiety of triacylglycerols (TAGs) affects the functional properties, as well as, the metabolism of oils (Angers & Arul, 1999).

TAGs are the main constituents (approximately 99 %) of frying oils, are the most abundant of lipid structures and are important storage lipids. They generally contain a mixture of different fatty acids. The structure of TAGs consists of fatty acyl groups esterified to a glycerol backbone by an ester bond (Ratledge & Wilkinson, 1988; Frankel, 1998).

The procedures for stereospecific analysis of TAGs, thus the determination of the compositions of the fatty acids located in each of positions *sn*-1, -2 and -3, tend to be quite complex and involve a number of chemical and enzymatic hydrolytic reactions as well as synthetic steps. The intermediates for each stage in the reaction should be isolated in a high degree of purity by chromatographic methods (Christie *et al.*, 1991; Christie, 1999). There are several different methods available to determine the positional distribution of TAGs of which some examples include, gas chromatography (GC), thin layer chromatography (TLC), chiral-phase or normal phase high-performance liquid chromatography (HPLC). However, methods used to determine positional distribution of

fatty acids in TAGs are time consuming which limits their use on a routine basis, or require analytical techniques which are prohibitive for general use. Gas chromatography (GC) is the most appropriate method by far for the analysis of fatty acids, generally in the form of methyl ester derivatives (Redden *et al.*, 1995a; Angers & Arul, 1999; Christie, 1999).

Literature describes methods that are used to carry out regio- or stereospecific analysis of fatty acids in TAGs which start with partial deacylation by pancreatic lipase, or by a Grignard reagent for the specific removal of a fatty acid (Redden *et al.*, 1995b; Angers & Arul, 1999). The two approaches yield answers that can be interpreted as, firstly, analysing 2-monoacylglycerols (MAGs), which are usually isolated by thin-layer chromatography, for their fatty acid composition and estimating values in the *sn*-1(3) position from the fatty acid composition of 2-MAGs and TAGs. The second approach involves analysing diacylglycerols (DAGs) for their fatty acid composition and determining the fatty acid composition in the *sn*-1(3) and *sn*-2 positions from the fatty acid composition of (DAGs) and TAGs (Angers & Arul, 1999).

The use of these methods on a routine basis is rather laborious, since isolation of either mono- or diglycerides is required when using HPLC. Another method, which may be used for regiospecific analysis of TAGs is tandem mass spectrometry and though very powerful, this is not a widely used method due to the limited availability of the instruments (Angers & Arul, 1999). A simple, fast, and reliable method for the regiospecific analysis of TAGs was developed by Angers and Arul (1999). Partial

deacylation of the TAGs by a Grignard reagent was carried out, followed by derivatization of the reaction products with n-butyryl chloride, in the presence of a base such as triethylamine, and direct analysis of the so-called dibutyrate derivatives of monoglycerides by gas chromatography.

It is important from the previous chapter to evaluate whether the stereospecific position of the EPOeq fatty acids is similar to that of plant Evening Primrose Oil and therefore it thus became the aim of this chapter to investigate the composition of EPOeq using quantitative techniques described in literature as well as additional quantitative methods such as NMR.

2. Materials and methods

2.1 Preparation of Grignard reagent

A 250 ml round-bottomed flask containing a magnetic stirrer bar was attached to a reflux condenser and a dropping funnel with the aid of a Claisen adapter. The dropping funnel was positioned directly above the flask and the condenser on the side arm of the adapter. Calcium chloride drying tubes were attached to the top of the condenser and to the dropping funnel. The assembled apparatus was dried using a flame. The dropping funnel was removed from the Claisen adapter and 0.10 mol of magnesium turnings was added through the opening as shown in Figure 1. The funnel was then replaced and 10 ml of anhydrous diethyl ether was added through it. A solution of 0.12 mol of methyl iodide (MeI) in 25 ml of anhydrous ether was prepared; the solution was swirled to achieve homogeneity, and was added to the dropping funnel. A 2 to 3 ml portion of the halide-

ether solution was added from the dropping funnel onto the magnesium turnings. A change in the appearance of the reaction mixture shown by the presence of a cloudy/chalky solution was seen followed by formation of small bubbles at the surface of the magnesium turnings which indicated the start of the reaction. Once the reaction has started, the ether was observed to reflux, an extra 20 ml portion of anhydrous diethyl ether was added to the reaction mixture through the condenser to dilute the reaction mixture and to minimize the coupling reaction. The rest of the halide-ether solution was added dropwise to the reaction mixture at a rate that was just fast enough to maintain a gentle reflux. The total addition time was 15 to 30 minutes. At the end of the reaction the solution had a tan to brown chalky appearance. The Grignard reagent was used as soon as possible after preparation.

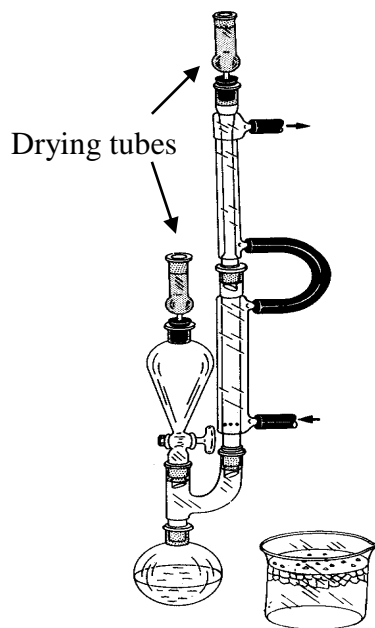


Figure 1. Apparatus for the synthesis of Grignard reagent (Experimental Organic Chemistry, Page 524).

2.2 Preparation of methyl esters

Methyl esters were prepared by adding 0.5 ml of methanol to 100 μ l of 1 g/l different fatty acid (FA) standards, Palmitic acid (C16:0), Palmitoleic acid (C16:1, [*cis*]-9), Stearic acid (C18:0), Oleic acid (C18:1, [*cis*]-9), Linoleic acid (C18:2, [*cis*]-9, 12) and Gamma-linolenic acid (C18:3, [*cis*]-6, 9, 12). The mixture was then reacted with diazomethane that was prepared by dissolving KOH (20 g) in distilled water (34 ml) followed by allowing the solution to cool down in a freezer after which ethanol (38 ml) was added. KOH (13.5 ml) and ethanol mixture was used for the preparation of diazomethane. A separate solution of diazald (3.75 g) in diethyl ether (34.5 ml) was prepared. The mixture reacted with diazomethane and was left for one hour at room temperature. Samples were then dried under nitrogen and stored at 4 °C (Pohl, 1999).

2.2.1 GC analysis of methyl esters

Methyl esters from Section 2.2 were dissolved in 200 μ l of chloroform and subjected to gas chromatography, with a flame ionisation detector and a Supelcowax 10 capillary column (30 m x 0.75 mm). The initial column temperature of 145 °C was increased by 3 °C/min to 225 °C and, following a 10 minute isothermal period, was increased to 240 °C at the same rate. The inlet and detector temperatures were 170 °C and 250 °C respectively. Nitrogen was used as a carrier gas at 5 ml/min. This analysis was done in triplicate and a response factor calculated, where peak area x factor = μ g/ μ l FA since a 1 μ l volume was injected with each analysis.

2.3 Preparation of ethyl ethers

For preparation of ethyl ethers, methyl esters as prepared in Section 2.2 were dissolved in 0.5 ml of anhydrous diethyl ether, which was contained in a flame-dried flask under inert atmospheric nitrogen. Equimolar amounts of ethyl magnesium bromide (Grignard reagent) were added in the same solution. After stirring at room temperature for 1 minute, 10 μ l of glacial acetic acid was added, followed by addition of 300 μ l of 10 % aqueous boric acid. The mixture was then extracted with 4 portions of diethyl ether saturated with boric acid. The organic extracts were combined, washed sequentially with 2 % sodium bicarbonate, water, and brine, dried over anhydrous sodium sulphate, filtered and concentrated under a stream of dry nitrogen. Samples were stored at 4 °C (Angers & Arul, 1999). Samples prepared were dissolved in 200 μ l of chloroform and subjected to GC analysis using a HP 4890A gas chromatograph equipped with a chrompack HT-SIMDIST CB column (10 m x 0.5 mm ID).

2.4 Lipid standards reaction with Grignard reagent

A DAG (Sigma, 99 % pure), 1,3-Dilinolein (C18:2, [*cis*]-9, 12) and a TAG (Sigma, 99 % pure), 1,2-Dioleoyl-3-stearoyl-*rac*-glycerol (C18:1, [*cis*]-9/ C18:1, [*cis*]-9/ C18:0) were reacted with the Grignard reagent over different time intervals, ranging from 1 minute up to 6 hours to determine at which optimum time interval FAs were being removed from *sn*-1 and 3 positions of the glycerol backbone. These samples were then subjected to NMR and GC analyses to verify the stereospecific arrangement of the FAs and as a validation of the stereospecific analysis of TAGs. EPO was also reacted with the Grignard reagent to confirm the *sn* position of Gamma-linolenic acid.

2.5 Spectroscopic methods

2.5.1 Nuclear magnetic resonance spectrometry (NMR)

NMR-spectrometry was performed on methyl esters, ethyl ethers and FA standards reacted with the Grignard reagent using a Bruker 300MHz DRX 300 spectrometer at 296K (23 °C). The solvent used was deuteriochloroform (CDCl₃). Chemical shifts are reported in parts per million (ppm) on the δ -scale and coupling constants are given in Hz. Chemical shifts of CDCl₃ are referenced δ 7.28 to TMS as the internal standard.

The following abbreviations are used:

s	singlet
d	doublet
dd	doublet-of-doublet
m	multiplet
br	broadened

2.6 Reaction of Neutral lipid (NL) fractions of various polymerised triglyceride containing oils with Grignard reagent

The neutral lipid (NL) fractions from the intracellular lipids (ICL) of *Mucor circinelloides f. circinelloides* grown on sunflower oil containing various PTG concentrations i.e. 5 % (w/w), 10 % (w/w), 15 % (w/w) and 45 % (w/w) were reacted with the Grignard reagent as described in Section 2.3 and subjected to GC analysis for

FA profile determination as discussed in Section 2.2.1 where FAs remaining on TAG were methylated by the addition of trimethyl sulphonium hydroxide (TMSOH) as described by Butte (1983). GC conditions were as described in Section 2.2.1.

3. Results and discussion

The method for stereospecific analysis of the FAs using the Grignard reagent and esterification coupled to specific reaction times were verified using both NMR and GC analysis. All modifications of FAs were verified by quantitative NMR and GC analysis. Figure 2 represents methylation of a fatty acid. The ^1H NMR (Figure 2a) of the 18:2 methyl ester (Figure 2b) revealed a residual deshielded singlet at $\delta 3.65$ integrating for 3 protons allocated to the *O*-methyl ester (H1'). The multiplet $\delta 5.40$, the triplet at $\delta 2.80$, the multiplet at $\delta 2.10$ and the triplet at $\delta 0.95$ (H18) were assigned to H-9 and -10, H-2, H-8 and -11 and H-18, respectively.

The completeness of the Grignard reaction was also confirmed by quantitative NMR. The ^1H NMR (Figure 3a) of the 18:2 methyl ester after extraction with equimolar amounts of the Grignard reagent (*O*-ethyl ether) (Figure 3b) showed a multiplet at $\delta 3.50$ integrating for 2 protons allocated to the H-1' of the *O*-ethyl ether. The multiplet $\delta 5.40$, the triplet at $\delta 2.80$, and the multiplet at $\delta 2.10$ were assigned to H-9 and -10, H-2, H-8 and -11, respectively. The multiplet at $\delta 0.95$ was assigned to H-2 and -18. The ^1H NMR spectrum (Figure 3a) showed absence of *O*-methyl protons at $\delta 3.65$ (*O*-methyl ester) and presence of a multiplet at $\delta 5.30$ (4-H, H-2') and doublets of doublets at $\delta 3.50$ (4-H, H-1' and 3') corresponding to the structure in Figure 3b. The rest of the peaks in the spectrum

were similar to those in Figure 2a thereby confirming the effectiveness of the Grignard reagent.

Results of lipid samples in Section 2.4 were analysed to confirm stereospecific removal of a fatty acid coupled to GC analysis to confirm stereospecific makeup of a lipid sample. Figure 4 shows the chemical structure of one of the lipid standards used for NMR analysis. The ^1H NMR spectrum of Figure 5a displayed multiples at $\delta 5.4$ (2H) and $\delta 5.3$ (1H) which were allocated to [*cis*]-9 double bond protons and H-2', respectively, corresponding to the structure in Figure 5b. The integration of 2H in Figure 5a instead of 4H in Figure 2a established hydrolysis of one of the unsaturated esters. In the same spectrum, the H-2 protons also integrate for 4 protons, which unambiguously confirms the *sn*-1 cleavage at 60 seconds. The presence of a triplet at $\delta 2.35$ (2H) in the ^1H NMR (Figure 6a) confirms that only two methylene protons at H-2 of the second chain are present. The protons at $\delta 4.25$ allocated to the [*cis*]-9 double bond also integrate for 2 protons, once again confirming the *sn*-1 and *sn*-2 cleavages after 75 seconds indicated in the compound in Figure 6b. This was substantiated by presence of a multiplet at $\delta 2.0$ in the same spectrum integrating for 4-protons allocated to H-7 and -8 methylene protons. After 120 seconds, only the fatty acid protons were observable in the spectrum (Figure 7a). Total hydrolysis was evident from absence of the methylene protons at $\delta 4.35$, which indicates that the glycerol moiety was totally cleaved. The spectrum (Figure 7a) corresponds to the structure in Figure 7b. This confirms the analysis of choice and further samples were subjected to the same standard methodology and FA compositions quantified by GC.

The same standard samples on which NMR analysis was carried out were subjected to GC analysis as described in Section 2.2.1. The molar ratio of 18:1 (64 %) to 18:0 (36 %) at reaction time zero was confirmed to be 2:1. As the reaction proceeded, confirmation at time 75 seconds showed only 18:1 (100 %) remained from the standard confirming NMR results obtained.

The PTG make up of the oils with varying concentrations in Chapter 3 consist of different TAG species within each PTG group. It is important to note that all the TAG species were analysed relative to the composition, and the TAG species were not separated before stereoselective analysis started. Table 1 shows the fatty acid ratios of the unreacted PTG containing oils (5 %; 10 %; 15 % and 45 % w/w) and EPO; it gives an indication of the stereospecific rendering of FAs on various PTGs. EPO as well as PTG containing oils were reacted with the Grignard reagent. With EPO, an increase in ratio for 16:0 was seen after reaction 1 indicating a large % is not at *sn*-1 followed by a decrease after reaction 2 indicating that a certain % of this fatty acid is esterified at the *sn*-2 position while the rest is retained at *sn*-3. The 16:1 fatty acid showed an increase in ME ratio after reaction 1 and 2 indicating that this fatty acid is found overall at *sn*-2. A decrease in the ratio of 18:0 was seen after both reactions indicating that this fatty acid is esterified at the primary positions. A decrease in ratio after reaction 1 was seen with 18:1, indicating a large % of this fatty acid is esterified at *sn*-1 followed by an increase after reaction 2 suggesting that most of this remaining fatty acid is esterified at the *sn*-2 position. The 18:2 fatty acid showed a decrease in ratios after both reactions indicating its *sn* position to be at the primary positions. The 18:3 fatty acid showed an increase in

ratios after the reactions with the Grignard reagent and no further change for the second reaction indicating that this fatty acid is esterified at the *sn*-2 position. This is similar to literature where research carried out on EPO showed that GLA is found mostly at the *sn*-2 and *sn*-3 positions (Christie *et al.*, 1991; Angers & Arul, 1999).

For the 5 % (w/w) PTG oil, there was an increase in the ratio of 16:0 after the first reaction with the Grignard reagent followed by a decrease in the ratio after the second reaction. This indicates that after the first reaction, 16:0 is mostly at *sn*-2 and 3 (initial reaction removes primary positions indicating that 16:0 remained on glycerol backbone) and after the second reaction (which indicated relative FAs were removed from the *sn*-3 position), 16:0 is indeed removed from the primary positions but a percentage remained after the final reaction time of 75 seconds confirming that the 5 % (w/w) PTG oil has 16:0 at the *sn*-2 position. For 16:1, there is an increase after both reactions, which indicates that this fatty acid is mostly retained at *sn*-2. With the 18:0 fatty acid, the very small change in the relative % ratio after reaction 2 confirms the primary *sn*-1 theory. For 18:1 fatty acid, the increase in the relative % ratio after reaction 1 shows that it is esterified at the *sn*-2 and -3 positions. Very little change was observed after the second reaction which shows that a large fraction of 5 % (w/w) PTG oil has 18:1 as the fatty acid esterified at *sn*-2. With the 18:2 fatty acid, the decrease in the relative % ratios after reaction 1 shows that this fatty acid is primarily found at *sn*-1. The further decrease in ratio after reaction 2 shows that this fatty acid is esterified more at the primary positions. However it is important to note that a % of the total species is bound to the *sn*-2 position. With 18:3, a decrease in ratio was observed after the first reaction indicating that a % of

this fatty acid is esterified at *sn*-1 followed by an increase in ratio which shows that of the remaining 18:3 fatty acid most is esterified at the *sn*-2 position of some 5 % (w/w) PTG species.

The 16:0 and 16:1 fatty acids of the 10 % (w/w) PTG oil showed a similar pattern to that of the 5 % (w/w) PTG oil. With the 18:0 of the 10 % (w/w) PTG oil, a further decrease was observed after reaction 2 indicating that this fatty acid is esterified at the primary position. The 18:1 fatty acid of the 10 % (w/w) PTG oil showed a similar pattern to that of the 5 % (w/w) PTG oil after reaction 1, however a further increase in the relative % ratios was seen after reaction 2 confirming the esterification of this fatty acid at the *sn*-2 position. The 18:2 fatty acid of the 10 % (w/w) PTG oil showed a similar pattern to that of the 5 % (w/w) PTG oil concluding that most of this fatty acid is esterified at the *sn*-1 position of the glycerol backbone. The 18:3 fatty acid showed an increase in the relative % ratios after both reactions thus indicating that *sn*-2 is the favoured position for this fatty acid.

For the 15 % (w/w) PTG oil, the 16:0 fatty acid showed a decrease after reaction 1 followed by an increase after the second reaction, indicating that the primary position, *sn*-1 is initially preferred but reaction 2 shows a large % of this fatty acid remains on position 2. The 16:1 fatty acid of the 15 % (w/w) PTG oil showed a decrease after the first reaction indicating that this fatty acid is esterified at the *sn*-1 position, followed by a rather huge increase in the relative % ratio after the second reaction showing that most of this fatty acid is found relative to the other fatty acids at the *sn*-2 position. With the 18:0

fatty acid, an increase was seen in the relative % ratios after the first reaction confirming that the *sn*-2 or *sn*-3 positions are the preferred positions for this fatty acid. However a huge decrease in ratio after reaction 2 shows almost all of this fatty acid remained on the *sn*-3 position. The 18:1 fatty acid showed very little change in ratios indicating equal distribution at all positions of the glycerol backbone. The 18:2 fatty acid showed an increase in ratio after reaction 1 indicating that this fatty acid is esterified mostly at *sn*-2 and -3 of the glycerol backbone, followed by a decrease in ratio after the second reaction showing the primary position as the preferred position for this fatty acid. The 18:3 fatty acid of the 15 % (w/w) oil showed a similar pattern to that of the 5 % (w/w) PTG oil confirming that a certain % of this fatty acid is esterified at the *sn*-2 position.

With the 16:0 fatty acid of the 45 % (w/w) PTG oil, an increase in ratios was seen after both reactions, confirming that the *sn*-2 position is preferred for this fatty acid. The 16:1 fatty acid showed a similar pattern to that of the 5 % (w/w) and the 10 % (w/w) PTG oils. The 18:0 fatty acid showed an increase in ratio after reaction 1 indicating that it is esterified at *sn*-2 and *sn*-3 positions of the glycerol backbone, which differs from that of the 5 % (w/w) and 10 % (w/w) PTG oils. For the 18:1 fatty acid, a decrease in ratios was observed after both reactions, which is similar to the 15 % (w/w) PTG oil but differs from the 5 % (w/w) and 10 % (w/w) PTG oils posing the question whether higher concentrations of PTGs can be used for production of EPOeq. The 18:2 fatty acid of the 45 % (w/w) PTG oil showed a similar pattern to that of the 5 % (w/w) and the 10 % (w/w) PTG oils. The 18:3 fatty acid of the 45 % (w/w) PTG oil showed a similar pattern

again confirming that this fatty acid is esterified at the *sn*-2 position of the glycerol backbone.

This study showed that in general 18:2 and 18:3 are found at the same *sn* positions in oils with varying PTG concentrations. However other FAs such as 16:0 are found at different *sn* positions in oils with varying amounts of PTGs. The main FA comparison between EPO and PTG oils is reiterated in this chapter and as discussed in chapter 3. The *sn* distribution of PTG oils below regulatory limits mimics that of plant EPO, especially the lower percentage PTGs oils, with respect to the starting TAG distribution. With the higher PTG contents, the FAs on the *sn*-2 position differ showing that oils close to and above regulatory limits are less suitable for the production of EPOeq.

4. Conclusions

1. NMR and GC analysis have proven to be valuable analytical techniques for the stereospecific analysis of fatty acids.

2. Stereospecific analysis of oils with varying PTG concentrations shows that the stereospecific positions of some FAs vary with different PTG concentrations whereas certain FAs are found at the same *sn* position. Most significantly the 18:2 was found to be esterified at the *sn*-1 position of the glycerol backbone and 18:3 at the *sn*-2 position in all the oils with varying amounts of PTGs. Research carried out on EPO showed that GLA is found preferentially at the *sn*-2 and *sn*-3 positions. Our findings with the positional distribution of EPOeq were similar to that of EPO.

3. The study has shown that the 5 % (w/w) and 10 % (w/w) PTG oils had similar fatty acid profiles and also similar *sn*-distribution profiles to that of EPO. However, the PTG oils had more 18:1 on the *sn*-2 position whereas 18:3 was an abundant FA in EPO. With increasing PTG concentrations of 15 % (w/w) and 45 % (w/w) the fatty acid profiles and the *sn*-distributions were less similar to that of EPO.

5. Acknowledgements

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6. References

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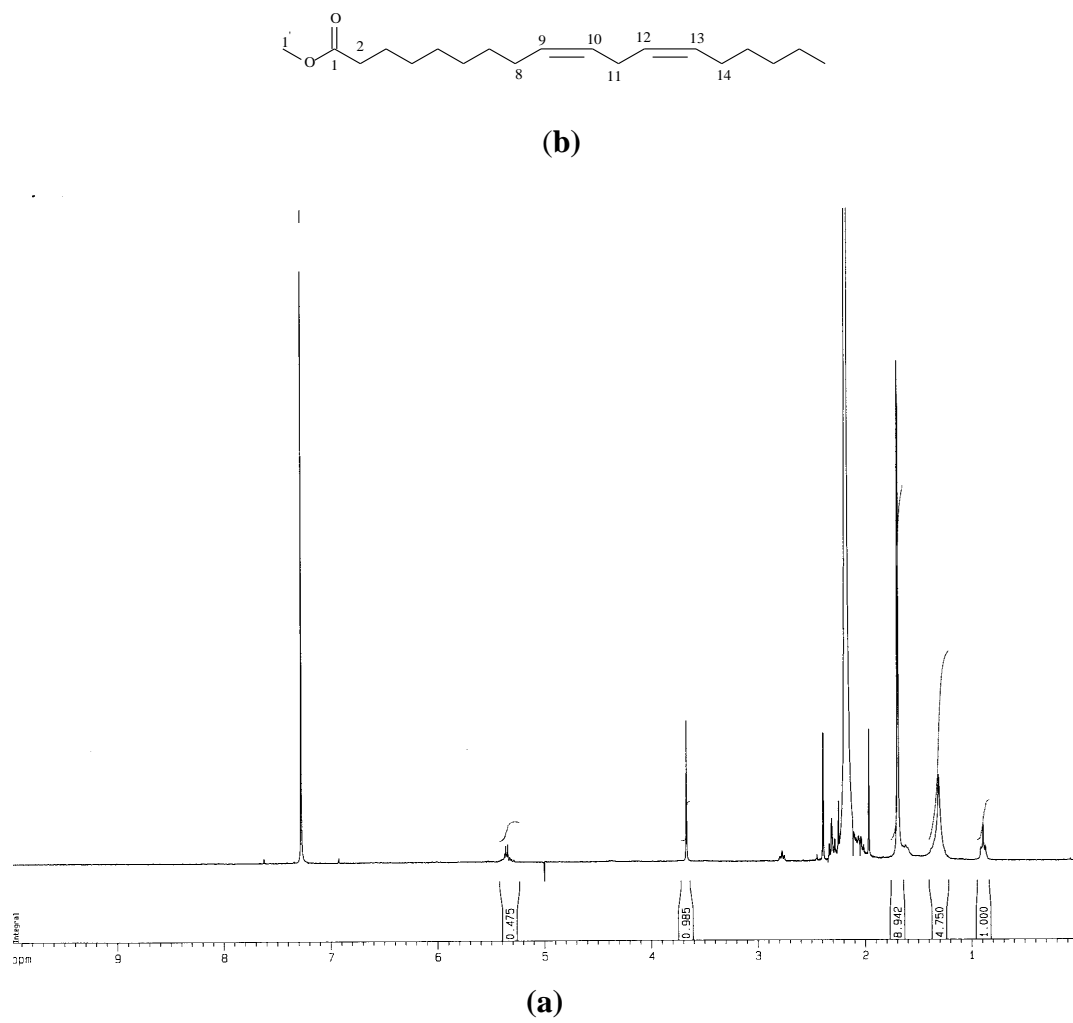


Figure 2a & b. ¹H-NMR spectrum and corresponding chemical structure of C18:2 methyl ester.

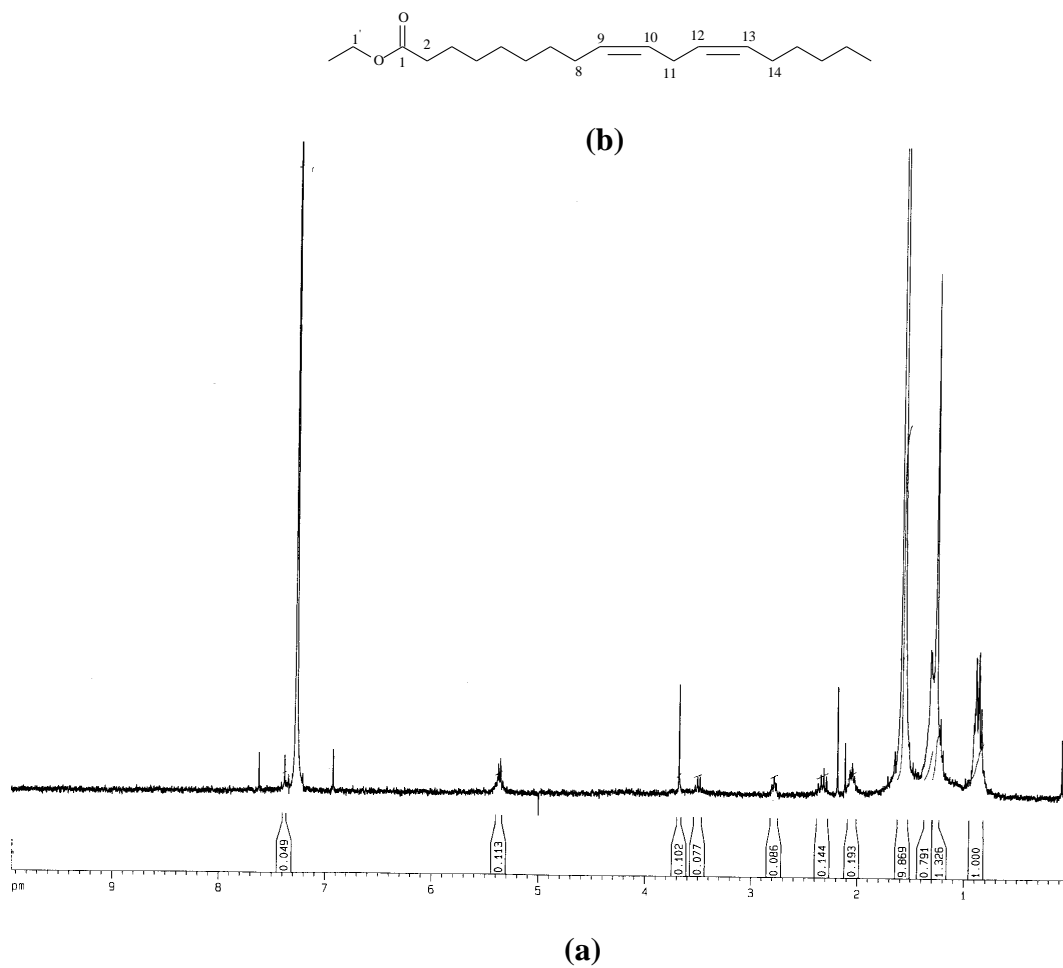


Figure 3a & b. ¹H-NMR spectrum and corresponding chemical structure of C18:2 methyl ester after extraction with the Grignard reagent.

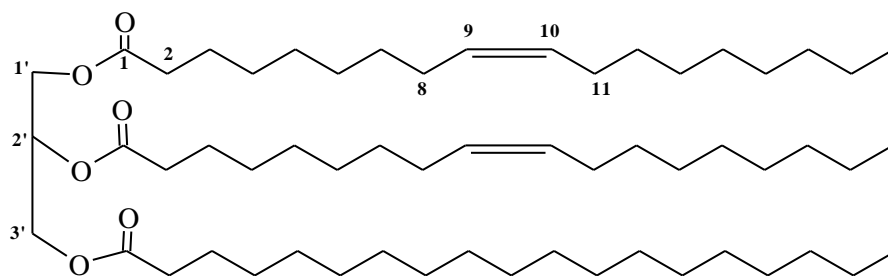
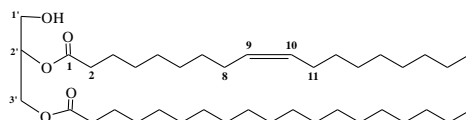
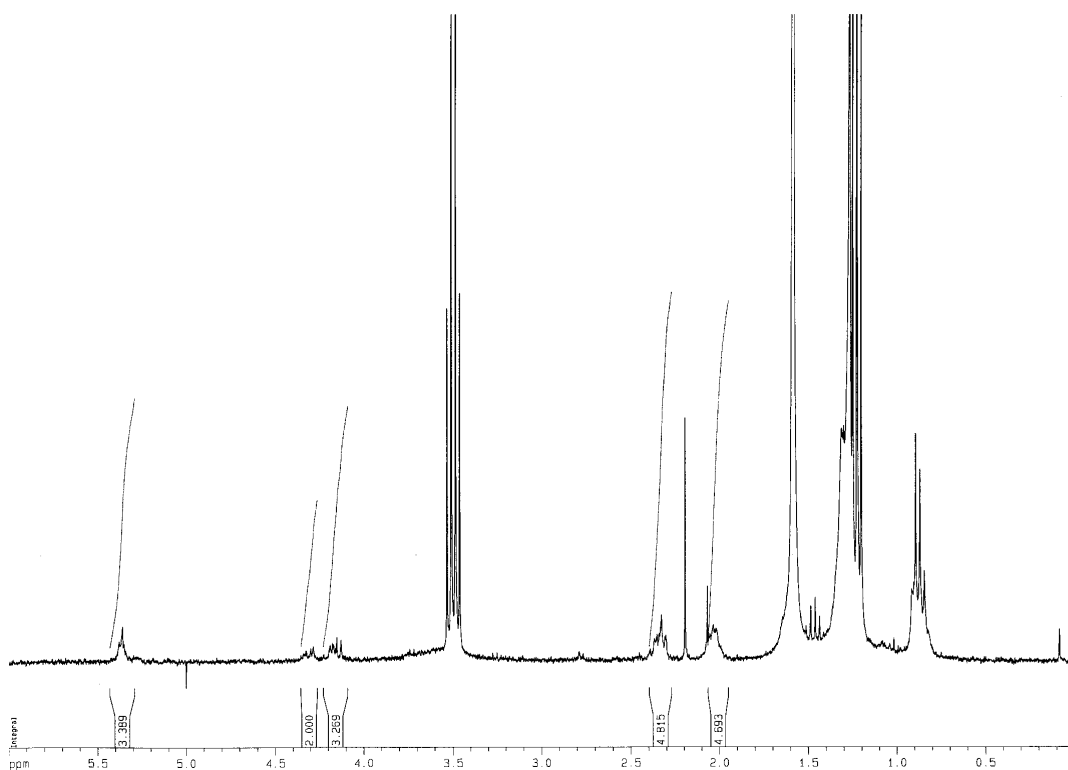


Figure 4. 1,2-Dioleoyl-3-stearoyl-rac-glycerol (C18:1, [*cis*]-9/ C18:1, [*cis*]-9/ C18:0).

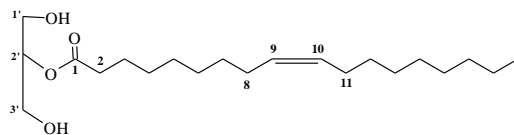


(b)

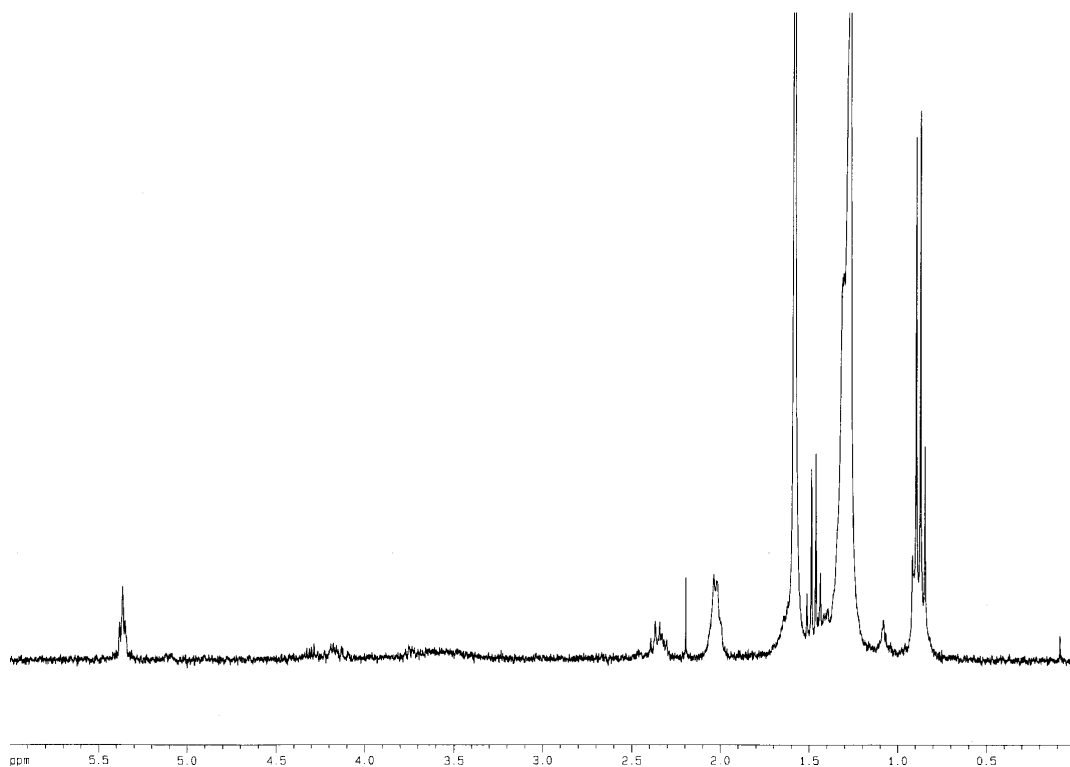


(a)

Figure 5a & b. $^1\text{H-NMR}$ spectrum and corresponding chemical structure of 1,2-Dioleoyl-3-stearoyl-rac-glycerol after 60 seconds reaction with the Grignard reagent.

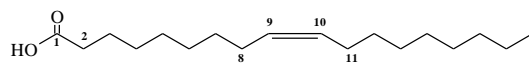


(b)

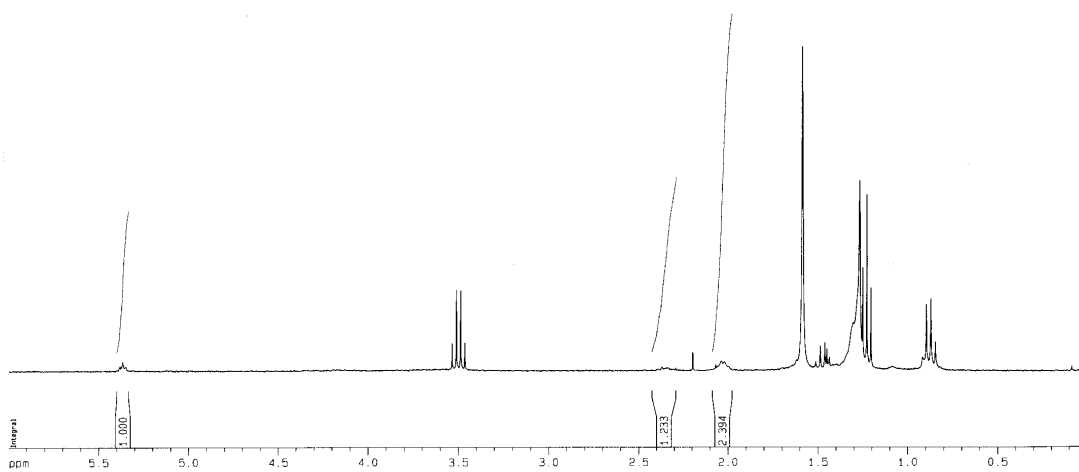


(a)

Figure 6a & b. $^1\text{H-NMR}$ spectrum and corresponding chemical structure of 1,2-Dioleoyl-3-stearoyl-rac-glycerol after 75 seconds reaction with the Grignard reagent.



(b)



(a)

Figure 7a & b. $^1\text{H-NMR}$ spectrum and corresponding chemical structure of 1,2-Dioleoyl-3-stearoyl-rac-glycerol after 120 seconds reaction with the Grignard reagent.

Table 1. Esterified FAs of PTG containing oils and EPO after reaction with the Grignard reagent. The remaining FAs were analysed by GC after transesterification.

FAs	PTGs 5 %			10 %			15 %			45 %			EPO		
	URO	<i>sn</i> -2 and 3	<i>sn</i> -2	URO	<i>sn</i> -2 and 3	<i>sn</i> -2	URO	<i>sn</i> -2 and 3	<i>sn</i> -2	URO	<i>sn</i> -2 and 3	<i>sn</i> -2	URO	<i>sn</i> -2 and 3	<i>sn</i> -2
16:0	2.6	9.8	4.7	0.9	11.2	5.9	14.4	8.4	14	1.5	10.6	15.5	2.9	7.1	4.1
16:1	0.1	1.1	5.9	0.1	0.9	5.8	0.6	0.1	5.6	0.1	0.3	5.9	0.2	0.9	5.9
18:0	4.9	1.5	1.3	6.9	3.8	1.3	5.9	6.4	0.9	4.6	6.6	1.1	5.2	3.3	2.0
18:1	15.3	32.1	31.6	24.5	28.1	31.1	26.8	26.1	25.8	32.8	30.2	26.9	17.1	8.9	11.3
18:2	76.3	55.5	52.5	67.2	53.0	51.9	50.4	58.6	49.8	60.5	51.9	47.5	73.8	70.9	68.0
18:3	0.7	0.1	3.9	0.5	3.0	3.9	1.8	0.4	4.0	0.5	0.3	3.0	0.7	8.9	8.6

EPO Evening Primrose Oil;

PTGs Polymerised triglycerides

Sn Stereospecific numbering

URO Unreacted oil

Summary

Approximately 100 000 tonnes of edible frying oil and fat waste, mainly derived from sunflower oil, is produced each year from the estimated 54 000 frying establishments in South Africa. Many of these establishments overuse or abuse their oils to save money. Such practices may result in oil breakdown and the production of harmful compounds, which can cause diseases such as cancer and diarrhoea when consumed. As a result, approximately 30 % of frying oil and fat waste in S.A. can be regarded as unhealthy while the other can be considered still useful for human consumption containing within S.A. regulatory limits i.e. equal to or less than 16 % polymerised triglycerides (PTGs) and/or equal to or less than 25 % polar compounds (PCs). In order to ensure that only oils and fats fit for human consumption are used during frying processes in S.A., it is important that sound quality control procedures are applied to all sectors of the oil industry. This is of special significance when taking into consideration the numerous cases of frying oil misrepresentations, adulteration and overuse reported over the years in S.A. The approximately 70 % of oil wastes still within the regulatory limits have the potential to be processed to safe usable foodstuffs such as Evening Primrose Oil equivalents (EPOeq). Evening Primrose Oil (EPO) has received attention over years as a medicinal supplement due to its high content of gamma-linolenic acid (GLA). A well-known fact is that the positional distribution of GLA on the glycerol moiety of triacylglycerols has an effect on its functional properties as well as metabolism. When *Mucor circinelloides f. circinelloides* CBS 108.16 was cultivated on a mixed substrate of sunflower oil (30 g/l) containing 1 %, 5 %, 10 %, 15 % and 45 % (w/w) PTGs and sodium acetate (10 g/l) as carbon sources, most of the oil was utilized after seven days of growth. Marked increases in biomass production as well as in the lipid content of the

fungal cells were also noted. Strikingly, these sunflower oils were transformed to fungal oils containing GLA and substantial amounts of linoleic acid (18:2) - also characteristic of evening primrose oil. This phenomenon was however not observed in the medium containing only oils with variable amounts of PTGs (40 g/l) and no acetate. In the presence of acetate, there was an increase in the pH of the medium from pH 5.8 to about neutrality as well as consumption of PTGs from the growth medium whereas in the absence of acetate there was a decrease in the medium pH from pH 5.8 to around pH 3.0 while the relative amounts of PTGs kept increasing in the extracellular lipids. The highest amounts of fungal oils were produced by *Mucor circinelloides f. circinelloides* using the 5 % and 10 % (w/w) PTG oils. This study suggests the production of EPOeq from sunflower oil waste within regulatory limits using *Mucor*. Stereospecific analysis of fungal oils obtained from oils with varying PTG concentrations show that the stereospecific positions of some fatty acids (FAs) vary with different PTG concentrations whereas certain FAs are found at the same *sn* position. The 5 % (w/w) PTG oil yielded a similar FA profile and also a similar *sn*-distribution profile to that of EPO, however with increasing PTG concentrations of 10 % (w/w), 15 % (w/w) and 45 % (w/w) the FA profiles and the *sn*-distributions were less similar to that of EPO. Gamma-linolenic acid was found at the *sn*-2 position in all the oils with varying amounts of PTGs. According to literature, GLA is found preferentially at the *sn*-2 and *sn*-3 positions in EPO. Our findings with the positional distribution of EPOeq were similar to that of EPO. Both Nuclear magnetic resonance (NMR) and Gas chromatography (GC) analysis have proved to be extremely valuable tools in the stereospecific analysis of FAs.

Opsomming

Ongeveer 100 000 ton eetbare kookolie- en vetafval, hoofsaaklik afkomstig vanaf sonneblomolie, word elke jaar geproduseer deur ongeveer 54 000 braairestaurante in Suid-Afrika. Baie van hierdie restaurante oorgebruik of misbruik hulle olie om geld te spaar. Sulke praktyke veroorsaak olie-afbraak en die produksie van skadelik verbindings wat siektes soos kanker en diaree kan veroorsaak as dit ingeneem word. Dus kan ongeveer 30 % van kookolie- en vetafval in S.A. beskou word as skadelik terwyl die res gesien kan word as bruikbaar vir menslike gebruik aangesien dit steeds binne die S.A. regulatoriese limiete val, d.i. gelyk aan of minder as 16 % gepolimeriseerde trigliseriede (PTGs) en/of gelyk aan of minder as 25 % polêre komponente (PKs). Om te verseker dat slegs olies en vette wat geskik is vir menslike gebruik in braaiprosesse in S.A. gebruik word, is dit belangrik dat goeie kwaliteitsbeheerprosedures in alle afdelings van die olie-industrie toegepas word. Dit is veral belangrik as die verskeie gevalle van kookoliewanvoorstelling, -vermenging en oorgebruik wat oor die jare in S.A. gerapporteer is, inageneem word. Die ongeveer 70 % olie-afval wat steeds binne limiete is, het die potensiaal om na veilige, bruikbare voedingstowwe soos Aandblomolie-ekwivalente (AOekw) verwerk te word. Aandblomolie (AO) het oor die jare baie aandag geniet as 'n mediese aanvulling as gevolg van 'n hoë gamma-linoleensuur (GLS) inhoud. Dis bekend dat die posisionele verspreiding van GLS op die gliseroleenheid van triasielgliserole 'n effek het op die funksionele eienskappe en metabolisme. *Mucor circinelloides f. circinelloides* CBS 108.16 is gekweek in gemengde media met sonneblomolie (30g/l) wat 1 %, 5 %, 10 %, 15 % of 45 % (m/m) PTGs bevat het, asook natriumasetaat (10g/l) as koolstofbronne. Meeste van die olie is gebruik na sewe dae van

groeï. Merkbare toenames in biomassa-produksie asook lipiedinhoud van funguselle is opgemerk. Die sonneblomolies is getransformeer na fungusi-olies met GLS en merkbare hoeveelhede linoleïensuur (18:2) - ook kenmerkend van AO. Hierdie verskynsel is egter nie waargeneem in die medium wat slegs olie met verskillende hoeveelhede PTGs (40 g/l) bevat het en geen asetaat nie. In die teenwoordigheid van asetaat, is 'n toename in die pH van die medium, vanaf pH 5.8 na ongeveer neutraal, asook 'n verbruik van PTGs uit die groeïmedium waargeneem. In die afwesigheid van asetaat was daar egter 'n afname in die medium se pH, vanaf pH 5.8 na pH 3.0, terwyl die relatiewe hoeveelhede PTGs in die ekstrasellulêre lipiede toegeneem het. Die grootste hoeveelheid fungusi-olie is geproduseer deur *Mucor circinelloides f. circinelloides* op 5 % en 10 % (m/m) PTG-bevattende olies. Die studie dui daarop dat die produksie van AOekw vanaf sonneblomolieafval binne regulatoriese limiete deur *Mucor* moontlik is. Stereospesifieke analises van fungusi-olie, verkry vanaf olie met verskillende PTG-konsentrasies toon aan dat die stereospesifieke posisies van sommige vetsure (VSe) variëer met verskillende PTG-konsentrasies, terwyl ander VSe in dieselfde *sn*-posisie aangetref word. Die 5 % (m/m) PTG-olie het 'n soortgelyke vetsuurprofiel en *sn*-verspreiding gelewer as AO. Met toenemende PTG-konsentrasies van 10 % (m/m), 15 % (m/m) en 45 % (m/m) het die vetsuurprofiel en *sn*-verspreiding egter minder ooreenstemming getoon met AO. Gamma-linoleensuur is in die *sn*-2 posisie gevind in alle olies met verskillende hoeveelhede PTGs. Volgens literatuur word GLS meestal in die *sn*-2 en *sn*-3 posisies in AO aangetref. Ons bevindinge aangaande die *sn*-verspreiding van AOekw is soortgelyke aan die van AO. Beide kernmagnetiese resonansie (KMR) en gaschromatografie (GC) analises was waardevolle in die stereospesifieke analises.

Keywords:

Evening Primrose Oil; Evening Primrose Oil equivalents; Gamma-linolenic acid; Malpractices; *Mucor circinelloides f. circinelloides*; Nuclear Magnetic Resonance; Oil waste; Polymerised triglycerides; Stereospecific analysis; Sodium acetate

Sleutelwoorde:

Aandblomolie; Aandblomolie-ekwivalente; Gamma-linoleensuur; Wanpraktyke; *Mucor circinelloides f. circinelloides*; Kernmagnetiese resonansie; Olie-afval; Gepolimeriseerde trigliseriede; Stereospesifieke analises; Natriumasetaat

