OPTIMIZATION OF PROCEDURES FOR OIL EXTRACTION FROM ANIMAL TISSUE

By

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DEDICATION

To my parents Dr and Mrs I.O Amusan and my siblings Tolulope, Fiyinfoluwa, Oluwadamilola and Precious Moyinoluwa

ABSTRACT

Oil was extracted from chicken and pork fat discards by microwave assisted extraction and soxhlet extraction for comparison. Protease enzyme and pulsed electric field were used as a pretreatment prior to extraction by either soxhlet or microwave assisted extraction by hexane. Conditions for both the enzyme pretreatment and the microwave extraction were optimized for the extraction of the oils from the discards. The fat discards were pretreated with papain enzyme in a phosphate buffer solution (pH 7.0) and incubated at 45^{o}C in a water bath and incubated at various time intervals (30 min, 1 h, 2 h and 3 h). Enzyme pretreatment at 2 h produced the highest oil yield increase of 20.1% when compared to the control samples (P=0.0003) in chicken samples and an oil yield increase of 16.6% in comparison to control samples (P=0.0179) in pork samples. There was a decrease in oil yield at 3 h and no significant difference in oil yield when compared to control in both chicken samples (P=0.217) and pork samples (P=1.0000). Optimization of microwave assisted process shows that microwave irradiation for 7.5 min produced the best oil yield and was the most effective in oil extraction from the animal discards. There was no significant difference in oil yield produced when the irradiation time was increased to 10 min, 12.5 min, and 15min. The results obtained from optimization of microwave assisted extraction were comparable to those obtained by soxhlet extraction for 3 hr. There were significant difference between oil yield obtained from both extraction methods (P=<0.05). There were no differences in the FAME profile when compared to the literature. Pulsed electric field pretreatments were carried out on pork samples at electric field strength of 2 kV cm^{-1}, 4 kV cm^{-1}, 6 kV cm^{-1}. The oil yields obtained were compared to values obtained from control samples after extraction using microwave assisted extraction for 3 min. Pulsed electric field at 4 kV cm^{-1} produced an oil yield increase of 6.79% and significantly different (P = 0.0344) compared with control samples. There were no significant differences between oil yield at 2kV and control samples (P=1.000). At pulsed electric field strength of 6 kV cm^{-1} there was a decrease in oil yield. The oil yields obtained from pulsed electric field pretreament were compared with that obtained by soxhlet extraction. Pretreatment at 4kv cm^{-1} gave an oil yield increase of 6% (P=0.1411). The quality assessment of the oil extracted with PEF pretreated samples showed no effect of PEF on the quality of the oil. There were no significant differences in the hydroperoxide

and p-anisidine values within the pretreatments and the control samples. PEF pretreatment had no effect on the fatty acid methyl ester (FAME) profiles of the oil.

RESUME

Les matières grasses des déchets associés au parage provenant des industries porcine et avicole ont été extraites par l'utilisation d'une extraction assistée par les microondes et par une méthode standard, la méthode Soxhlet. Deux prétraitements, l'hydrolyse enzymatique et le champ pulsé, ont été utilisés avant l'extraction à l'hexane des matières grasses. Les conditions d'application du prétraitement enzymatique et de l'extraction assistée par les microondes ont été optimisées pour maximiser l'extraction à partir des matières grasses rejetées par l'industrie. Les déchets gras ont été prétraités avec une protéase, la papaïne, dans un tampon phosphate (pH 7.0) et incubés à 45^{o}C dans un bain-marie pour des intervalles de temps variés (30min, 1 h, 2 h et 3h). Les augmentations les plus importantes de l'extraction des matières grasses ont été obtenues avec le prétraitement enzymatique des échantillons de volaille (20.1%, P=0.0003) et de porc (16.6%, P=0.0179) pour une durée de 2 h lorsque comparé aux échantillons témoins. Une diminution du rendement de l'extraction a été notée lors du traitement enzymatique d'une durée de 3h sans que ces valeurs montrent des différences significatives avec les échantillons témoins de volaille (p=0.217) et de porc (P=1.000). L'optimisation du procédé d'extraction par microonde a montré que l'irradiation avec les microondes pendant 7.5 min a produit les meilleurs rendements d'extraction. Aucune différence significative n'a été retrouvée lorsque les échantillons ont été soumis à l'irradiation pour des temps accrus à 10 min, 12.5 min et 15 min. Les résultats obtenus pour l'optimisation de l'extraction assistée par les microondes sont comparables à ceux obtenus par l'extraction pendant 3 h avec la méthode Soxhlet. Des différences significatives de rendement en matières grasses ont été obtenues avec les deux méthodes d'extraction (P=<0.05). Aucune différence n'a été retrouvée entre les profils d'esters méthyliques d'acides gras lorsque comparé avec ceux de la littérature. Le prétraitement impliquant l'application d'un champ pulsé a été utilisé sur des échantillons de porc à des différences de potentiel de 2 kV cm^{-1}, 4 kV cm^{-1} et 6 kV cm^{-1}. Les rendements en matières grasses ont été comparés aux valeurs témoins obtenues avec l'extraction assistée par les microondes pour une durée de 3 min. L'application de champs pulsés à 4 kV cm^{-1} a conduit à une augmentation significativement différente (P=0.0344) de 6.79% du rendement en matières grasses lorsque comparé aux échantillons témoins. Aucune différence significative n'a été retrouvée entre les rendements de l'échantillon témoin et l'échantillon soumis à un champ de 2 kV cm^{-1} (P=1.000). Le champ pulsé appliqué à un niveau

de 6 kV cm^{-1} a produit un rendement diminué en matières grasses. L'évaluation de la qualité des matières grasses extraites après un prétraitement avec des champs pulsés n'a montré aucun effet négatif des champs pulsés sur la qualité de la matière grasse. Aucune différence significative n'a été retrouvée sur les indices hydroperoxydes et *p*-anisidine entre les prétraitements et les témoins. Les champs pulsés n'ont, de plus, aucun effet sur le profil en esters méthyliques d'acides gras de la matière grasse.

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CHAPTER 1: INTRODUCTION

Oils and fats are mixture of lipids. According to Christie (2007), lipids comprise of fatty acids, their derivatives, and substances related biosynthetically or functionally to these compounds. They are mainly triacyglcerols and they also contain diacylglycerols, monoacylglycerols and free fatty acids, they also contain phospholipids, free sterols and sterol esters, tocols (tocopherols and tocotrienols), triterpene, alcohols, and hydrocarbons (Shahidi, 2004).

Extraction of oils from animal tissue has been historically carried out from bovine (tallow), swine (lard), and poultry (poultry grease or poultry fat) through the rendering process. The final product has edible or non-edible uses, such as for soap and lubricants production. Animal fat consists predominantly of glycerol esters of fatty acids and unsaponifiable matter (Hui, 1996). The distribution of fat between different sites of the animal varies with animal species, breed, degree of finish (Carrapiso and Carmen, 2000) and age, feeding habits and sex of the animal.

Lipids are present in tissues in different physical forms. Simple lipids are often part of a large aggregate in storage tissues, and can be extracted from this tissues with relative ease; complex lipids are however membrane constituents. They exist in close association with compounds such as proteins and polysaccharides with which they interact, and are therefore not easily extracted.

The Nutritional Labeling Act (NLA) of 1990 defines total fat content as including the sum of all fatty acids obtained from the overall lipid extract expressed as triglycerides. To obtain this, the

protocol described by the NLA involves a number of steps. This involves hydrolysis treatment of the source material by either acid, alkali or enzyme followed by solvent extraction of lipids, transmethylation using basic or acidic catalyst and individual separation of fatty acid methyl esters (FAMEs) by chromatography and quantification of the free fatty acid by HPLC (Shahidi, 2005)

Rendering is the process which is mostly used to extract fats and oils from animal origin. The aim of rendering is to obtain complete separation of fatty tissue components. Rendering systems rely on heat to release fat from the cells of the fatty tissues. The rendering process can be either wet rendering or dry rendering. In wet rendering, either boiling water or steam is added to the material causing fat to rise to the surface, then pressed to remove a water-fat mixture which is then separated into fat, water and fine solids by stages of centrifuging and/or evaporation. In dry rendering, fat is released by dehydrating the raw material. The material is first ground, and then heated to release the fat and drive off the moisture, percolated to drain off the free fat and remove the cooked proteinaceous residue (cracklings). If fatty tissues are handled and rendered properly, the resulting lard is suitable for use as food fat without further treatment. Edible animal fats may be subjected to bleaching, hydrogenation, deodorization,and/or intersterification to improve their characteristics.

Extraction of fats and oil for food use is mostly achieved through solvent extraction. Often times the oil seeds are pressed prior to solvent extraction. The oil recovered then goes through purification and deodorization steps before it can be used. However, for quantitative determination of fats and oils, other methods are used. The extraction solvent must be chosen to suit the tissue being analyzed. Solvents have different capacities and the lipid content of the tissue varies widely. Ether extraction is the classical method for lipid determination of feedstuffs (Hui, 1996). This includes solvents such as diethyl ether; petroleum ether and hexanes. Solvent extraction methods often produce large amounts of hazardous solvent wastes and are generally cumbersome. Automated extraction equipment such as the Soxhlet or Goldfish apparatus require long extraction times.

Methods used for the quantitative determination of total lipids and fatty acid composition in plant and animal tissues require solvent extraction (Klaus, 1998), usually organic solvents in mixtures such as chloroform and methanol, as in the Bligh and Dyer (1959); and Folch (1957) procedures. Other methods used for extraction and quantification of total lipids for analytical purposes are microwave extraction and supercritical fluids extraction (Jose and Gomes, 1985).
Due to environmental concerns and potential health hazards of organic solvents methods such as microwave extraction which use minimal amount of solvent are proposed. Microwave extraction makes use of the dielectric properties of the solvent used. Microwave energy, increases the rotational force on bonds connecting dipolar moieties to adjacent molecules, reduces the energy required to disrupt hydrophobic associations, hydrogen bonding and electrostatic forces, thus enabling the dissolution of all kinds of lipids in the sample. Microwave extraction technology is a rapid, cost effective and an environmentally safe method for extracting lipids. Microwave technology has also been applied to the digestion of samples for measuring trace metals, and for the extraction of organic compounds from various materials (Palmquist and Jenkins, 2003).

Supercritical fluid extraction involves the use of a gas (usually CO_2) subjected to temperatures and pressures over limits known as critical pressure and critical temperature. Above these points, the super critical fluids have different properties in the gaseous state; such as increased solvation power (Organization of World Intellectual Property, 1987). The use of supercritical fluid for lipid extraction significantly reduces the use of organic solvents, avoids waste disposal problems, eliminates the use of potentially toxic and flammable solvents and reduces the extraction time.

Enzymatic hydrolysis is amongst methods proposed to improve oil extraction from oil materials especially oil seeds. Enzymatic hydrolysis prior to solvent extraction has been proven to increase extractable oil from biological samples (Akoh and Min, 2002). The interest in animal fat is due to the need for alternative fuel such as biodiesel which is environmentally friendly. Investigation on the use of animal fat to produce biodiesel is ongoing. Use of animal fat discard is more cost efficient and eliminate the problem of waste fat disposal.

1.1 Study Rationale

The overall project objectives were to investigate the effect of enzymatic various pretreatment techniques on fat extraction and fat yield from animal (pork and chicken) processing discards using microwave assisted extraction versus conventional methods.

Specific Objectives: The specific objectives for the proposed study were:

To investigate the effect of enzymatic hydrolysis on fat yield from pork and chicken slaughtering discards;

To optimize the extraction of fat from pork and chicken slaughtering discards using microwave extraction; and

To investigate the effect of Pulsed Electric Field (PEF) on yield of fat from pork slaughtering discards using microwave assisted extraction and comparing it with conventional soxhlet extraction methods.

CHAPTER 2: LITERATURE REVIEW

2.1 CHEMISTRY OF LIPIDS

Fats and oils make up about 99% of the lipids of plant and animal origin (Hui, 1996). Fats and oils are distinguished by their physical state at room temperature. The term fat refers to the lipid mixture which is solid at room temperature, while oil refers to that which is liquid at room temperature. All fats and oils are mostly constituted by triacylglycerols. Triacylglycerol molecules have a glycerol backbone with up to three fatty acids esterified to it. A glycerol molecule with three fatty acids are called triacylglycerols (TAG); when two fatty acids are esterified to the glycerol it is called diacylglycerols(DAG); and if only one fatty acid is present, it is a monoacylglycerol (MAG) (Afaf and Jan, 2005).

Fatty acids are characterized by the number of carbons and degree of unsaturation in the molecule. Typical edible oils have fatty acids of carbon length ranging from 4 to 20. Fatty acids can be further separated into two categories namely, saturated and unsaturated fatty acids. Saturated fatty acids are those which contain no double bonds as each carbon atom is surrounded by other carbon atoms and hydrogen atoms. These fatty acid molecules are joined in a zig-zag chain as there is free rotation about the carbon atoms due to the absence of double bonds. Fat molecules with saturated fatty acids align easily to create a closely packed structure (Semih, 2005). Figure 1 is a typical structure of the 18 carbon chain fatty acids with one (mono-unsaturated), two, and three (polyunsaturated) double bonds as well as that of stearic acid, the corresponding saturated fatty acid.

Unsaturated fatty acids contain carbon to carbon double bonds. There are also two possible configurations for a double bond. Double bonds for which the hydrogen atoms are on the same side of the chain are “cis” double bonds, and those where hydrogen atoms are at opposite sides of the bond are called “trans”. These fatty acids can have one (mono-unsaturated), or more (poly-unsaturated) double bonds, due to the presence of one or more double bonds in unsaturated fatty acids creating kinks in the fatty acid carbon chain (Akoh and Min, 2005).

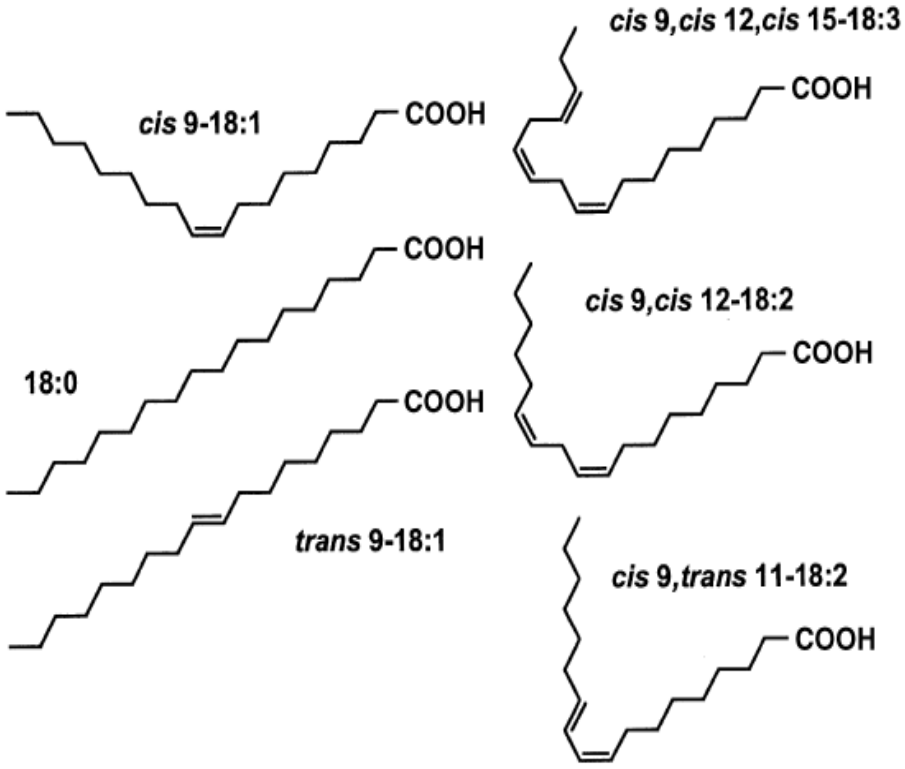

Figure 2. 1 Chemical structure for common fatty acids in oils and fats

The melting points of fatty acids depend on the chain length of the fatty acid as well as the degree of unsaturation of the molecule. For saturated fatty acids, the melting point increases as the chain length increases. The melting point of unsaturated *trans* fatty acids is greater than that of *cis* fatty acids of the same chain length, with the difference being as large as 20 ^{o}C for corresponding structures. The melting characteristic of TAGs depends on the composite of fatty acids and their positions on the glycerol backbone.

2.2 FUNCTIONS OF LIPIDS

Fats are also considered a source of energy and a building block of membranes. Fats are an important component of many food products as they contribute to the mouth feel (texture and lubricity) and volatility of the flavor compounds of the food product. Fats are used as shortening in baked goods; they provide tenderness, structural integrity, aid in the incorporation of air, and to extend shelf life of food (Harry, 1995).

Fats are also used in the production of confectionaries and as emulsifiers in food products. Dietary fatty acids of short and medium chain-length are not usually esterified but are oxidized rapidly in tissues as a source of 'fuel' to support all the events necessary to keep organisms functioning. Longer-chain fatty acids are usually esterified to glycerols in tissues (Shahidi, 2004).

Essential fatty acids are vital for growth, building cell walls and aid in the construction of phospholipids. Essential fatty acids are fatty acids that cannot be synthesised by the body and should therefore be obtained from the diet. They aid in lowering excessive cholesterol levels in the body, thereby decreasing the occurrence of cardiovascular diseases. Fatty acids are also the

biosynthetic precursors of many insect pheromones and secondary metabolites in plants (Maxwell and Marmer, 1983). Unesterified fatty acids can act as second messengers required for the translation of external signals, as they are produced rapidly as a consequence of the binding of specific agonists to plasma membrane receptors.

Within cells, fatty acids can act to amplify or otherwise modify signals to influence the activities of such enzymes as protein kinases, phospholipases, and many more. There are other uses and functions of animal fat. They are involved in regulating gene expression, mainly targeting genes that encode proteins with roles in fatty acid transport or metabolism via effects on transcription factors, i.e., peroxisome proliferator-activated receptors (PPARs) in the nuclei of cells. Such effects can be highly specific to particular fatty acids (Hui, 2006). Thus, unesterified arachidonic acid may have some biological importance as part of the mechanism by which apoptosis (programmed cell death) is regulated.

Stores of fat within the human body are important as they provide quick access to energy while fasting, act as insulation from hypothermia, while fat surrounding organs provides an internal layer of protective padding (Shahidi, 2004). The ingestion of fats however, must be done with moderation. It has been found that saturated fats are major causes of cardiovascular disease such as arthelesclerosis (Gunstone, 2004). They increase low density lipoprotein (LDL) cholesterol, the so called “bad” cholesterol, which leads to coronary heart disease. Trans fats, introduced during partial hydrogenation causes and increase in LDL cholesterol while concurrently decreasing HDL cholesterol (Gunstone, 2004).

2.3 SOURCES OF ANIMAL FAT

Fats from animal origin come from various sources such as chicken, pork, cattle, sheep, and other types of animals. The amount of fats found on these animals depends highly on their diet. The amount of fat found in the animal depends also on location on the animal. Major fat depots include the subcutaneous fat (located under the skin and overlying superficial muscles) and the intermuscular fat (located between muscles). Certain amounts of fat are also found deposited in the abdominal cavity of the animals. Animal species, breed and degree of finish greatly affect fat distribution on this various sites.

Poultry fats are produced from various bird species and their fatty acid composition depends largely on the food ingested. Chicken fat has a high proportion of palmitoleic (16:1 n-9) and linoleic acid (18:2 n-6). Fatty liver of duck or goose has triglycerides rich in oleic acid and small amounts of palmitic and stearic acid.

Tallow is fat rendered primarily from cattle, sheep and goats. Its fatty acid composition depends on the tissue location more than on the food ingested. The proportion of fat in the dressed weight varies from 8% up to 25% fat. Lard, the fat from pork comes from cutaneous adipose tissue of the pig. The fatty acid composition of lard depends on the diet of the pig. For example the linoleic acid content increases when fed with soya as compared to rice. The dressed weight of pigs contains about 30% fat, but very fat pigs can contain up to 50% fatty matter. Production of animal fat has increased over the past years due to higher demand for animal products.

Table 2. 1 shows the amount of fat produced from various groups of animals per year in the United States of America.

Fat deposits are also found around the kidney, heart, and intestines. This fat is removed from animals after slaughter. During production, there is a lot of floor killings, which are fat removed from the carcass when the animals are being dressed. Most often these fats are discarded as waste materials from the slaughterhouse or butchery, wholesale or retail outlets (such as grocery stores/ supermarkets).

Tallow is used in soap making and in production of animal feeds. Inedible tallow is also used in producing plastics, lotions, soaps and detergents, tires, candles, paints and varnishes, lubricants and several pharmaceuticals. Tallow is an important commodity in the global fat and oils market. These fats play an important role in the formulation of pesticides, herbicides, emulsifiers and dispersing agents. Several derivatives are produced from tallow: fatty alcohols, amines, amides, esters and glycerol (Akoh and Min, 2002).

2.4 OIL EXTRACTION

Oil has been extracted from several oil bearing materials such as seeds, fruits and nut for thousands of years. Various plant varieties produce oils in commercial quantities, thus emphasis has been placed on oil extraction technologies to maximize oil yields while minimizing the production of undesirable impurities during extraction (Carr, 1997).

Lipids are naturally associated with other molecules such as carbohydrates and proteins via van der Waals forces, electrostatic and hydrogen and/or covalent bonding. Because of their association with other food components, to separate and isolate lipids from a complex cellular matrix requires the use of different physical and chemical treatments (Harry, 1995). Solubility of lipids in organic solvent is the general property of lipid used in extraction from other cellular components. Total extraction of lipid involves long extraction times or a combination of solvent with different polarity for solubility of lipids from the matrix. Accurate and precise analysis of lipids in foods is important for determining constituting components and nutritive value, standardizing identity and uniformity, preparing nutritional labelling material, as well as for promoting and understanding the effects of fats and oils on food functionality (Gunstone, 2007)

Since animal fats, either from whole animals, depot fats, or viscera contain active enzymes, the fat should therefore be extracted as quickly as possible. Oils from whole fish or from fish waste are highly unsaturated and extraction should therefore be carried out rapidly because they are liable to rapid oxidation. Oils from fruits, such as those from the oil palm, have high moisture content and the oil should also be extracted as quickly as possible to minimise enzymic hydrolysis (Akoh and Min, 2008).

There are several important steps involved in oil extraction and they include the following:

2.4.1 Sample Preparation

Proper storage of samples and sampling are important in obtaining good and valid results. Adequate sample preparation procedure is required in order to obtain an ideal and uniform samples. An ideal sample should be identical in all of its intrinsic properties to the bulk of the material from which it is taken (Pomerranz and Meloan, 2000). Sample preparation of lipid for

analysis varies depending on the type of food and nature of its lipids and it requires adequate knowledge of the structure, chemistry and occurrence of principal lipid classes and their constituents. Oils from tissues of all origins should be extracted immediately after removal from the living organism to prevent changes to the lipid components (Christie, 2007). Since immediate

Table 2.1 Animal fat production in 1992

Animal fat	(Billion pounds/yr)
Edible tallow	1.625
Inedible tallow	3.859
Lard & grease	1.306
Yellow grease	2.633
Poultry fat	2.215
Total animal fat	11.638

Source: Pearl, 2002)

extraction is not always possible, the samples are usually stored in sealed containers under inert atmosphere at very low temperature (-35^{o}C) to minimize changes (Pomerranz and Meloan, 2000). The freezing process however can damage the tissue as a result of osmotic shock. Proper care should therefore be taken when storing tissue for further analysis.

For effective extraction of lipids from the tissue several pretreatments are necessary.

2.4.1.1 Drying

Drying is an important step prior to solvent extraction. Some non-polar solvent such as hexane do not easily penetrate tissues with high moisture content and therefore ineffective lipid extraction occurs. Drying reduces the moisture content of the samples and helps to enhance oil extraction from the material. Predrying facilitates grinding of sample to reduce particle size, enhance oil extraction and may break fat water emulsion to make fat dissolve easily in the organic solvent to help free tissue lipids (Akoh and Min, 2008). Different drying methods can be employed and this includes vacuum oven drying or freeze drying. Drying samples at high temperatures is not encouraged as the lipids become bound to proteins and carbohydrate and such bound lipids are not easily extracted with organic solvents and enhance oxidation (Hui, 1996).

2.4.1.2 Particle size reduction

Particle size reduction is also an important step in sample pretreatment. Extraction efficiency of lipids from dried samples also depends on the particle size. Particle size reduction promotes good solvent contact as it provides a greater surface to volume ratio, enhances oil extraction resulting

in higher oil yields. Flaking is another term used which is a refined grinding process which produces a uniform particle size (Akoh and Min, 2002). This involves rolling the crushed oil bearing materials into thin flakes of about 0.20 to 0.35mm thick. This is normally applicable to oil bearing materials such as seeds and fruits (Bockisch, 1997).

2.4.1.3 Hydrolysis

Food matrices are often treated with acid or alkali prior to extraction in order to make lipids in the food samples more available for the extracting solvent. Hydrolysis is used to release covalently and ionically bound lipids to proteins and carbohydrate as well as emulsified fats. The samples require digestion by 3-6 M hydrochloric acid (HCL) under reflux condition and converts bound lipids to an easily extractible form (Akoh and Min, 2008). Dairy food samples require alkali hydrolysis using ammonia to break emulsified fat, neutralize any acid and solubilize proteins before solvent extraction. Hydrolysis by use of enzyme is also employed to hydrolyze food carbohydrate and protein to make the lipids more available for solvent extraction. The use of enzyme as a means to improve oil extraction will be further discussed in detail.

2.7 METHODS OF OIL EXTRACTION

2.7.1 Solvent extraction

The insolubility of lipids in water makes it possible to separate them from proteins, carbohydrates and water in the tissues. Solvent extraction is defined as the process of separating liquid from a solid-liquid system using a solvent. Lipids have a wide range of hydrophobicity which depends largely on their molecular constituents (Akoh and Min, 2008). Lipids exist in tissues in different physical forms. In food analysis, fat content refers to lipids that can be extracted into less polar solvents such as light petroleum ether or diethyl ether (Gunstone, 2004).

Complex lipids are usually constituents of membranes, where they occur in a close association with other compounds such as proteins and polysaccharides, with which they interact; thus they are not extracted so readily. These bound lipids require more polar solvents, for their extraction.

Hexane is the common commercially used solvent (Christie, 2007). The use of solvents such as ethanol, isopropanol, methylene chloride and chloroform has been investigated. During solvent extraction, van der Waals bonds, electrostatic interactions and hydrogen bonds are broken to different extents; covalent bonds however remain intact (Shahidi, 2004).

Non-polar solvents can be used to extract neutral lipids hydrophobically bound while polar lipids bound by electrostatic forces and hydrogen bonding require more polar solvents capable of breaking such strong bonds. Triacylglycerides (TAG's) and cholesterol esters which are neutral lipids can be extracted to some extent by non polar solvents; the extraction is however incomplete which might be due to the inaccessibility of a significant part of the lipids to the solvents (Kemper 1997). Lipids bound to polypeptide and polysaccharide groups are not extracted at all by organic solvents and remain in the non lipid residue (Akoh and Min, 2008). This is why a hydrolysis step is often required to break the covalently bound lipids for easy extraction. In order to release all lipids from their association with cell membranes or with lipoproteins, the ideal solvent or solvent mixture must be fairly polar. However the solvent must not be so polar that it reacts chemically with the lipids or that triacylglycerols and other non-polar simple lipids do not dissolve and are left adhering to the tissues.

Solvent extraction involves introducing an oil bearing material into a bath of solvent (Kemper 1997). The oil bearing material is allowed to soak in the bath of solvent and allowed enough time for the solvent to dissolve the oil. Miscella is formed upon concentration of the oil in the solvent and the oil therefore migrates out of the material into the solvent bath (Gunstone, 2004). This process continues repeatedly until the oil and solvent reach an equilibrium with the particle and the miscella. The dissolution theory can be used to explain solvent extraction which is based on the laws of thermodynamics.

Two endothermic processes and one exothermic process are involved in dissolution during extraction. Energy is required in the process, first to break the solute into isolated molecules and secondly energy is required to break intermolecular bonds in the solvent to accommodate the solute. Energy required is small when the solute molecules are non polar. Exothermic reaction results from the interaction of solute and solvent which results in solution equilibrium. The

principle lies in the fact that non polar solutes are more soluble in non polar solvents and therefore non polar lipids are readily solubilized by non polar solvents such as hexane and petroleum ether (Akoh and Min, 2008).

2.7.2 Choice of solvent

In order to extract lipids from tissues, it is necessary to use solvents that not only dissolve the lipids readily but overcome the interactions between the lipids and the tissue matrix. No single pure solvent appears to be suitable as a general-purpose lipid extractant. The type of solvent and the actual method of lipid extraction depend on both the chemical nature of the sample and the type of lipid extract desired (Christie, 2007). An important characteristic of the ideal solvent for lipid extraction is the high solubility of lipids and non solubility of proteins, amino acids and carbohydrates. The solvent can also ensure the absence of side reactions by preventing enzymatic hydrolysis of lipid. The solvents should be heat, light and water stable and should not react with the oil, meal or any equipment used which can lead to toxicity problems (Hui, 1996). The type of solvent used should be highly pure to ensure uniform operation and extraction. The solvent should also not be highly flammable to reduce the risk of fire and explosion. The solvent used should readily penetrate sample particles and should have a relatively low boiling point to evaporate readily without leaving any residues when recovering lipids. The solvents mostly used for isolation of lipids are alcohols (methanol, ethanol, isopropanol, n-butanol), acetone, acetonitrile, ethers (diethyl ether, isopropyl ether), halocarbons and hydrocarbons (hexane, benzene) (Shahidi, 2004).

Solvents such as benzene are often avoided because of their potential carcinogenic effect. It is also important that the particle sizes of the sample allow for adequate solvent percolation while minimizing solvent diffusion. The reduction of particle size increases the surface area, decreases solvent penetration path lengths resulting in increased oil transfer rates into the solvent (Akoh and Min, 2002). The proportion of nonpolar hydrocarbon chain of the fatty acids or other aliphatic moieties and polar functional groups such as phosphate or sugar moieties in their molecules dictate the solubility of lipids in organic solvents. Lipids such as triacylglycerides (TAGs) or cholesterol esters are highly soluble in hydrocarbon solvents such as hexane and

benzene; they however are insoluble in polar solvents such as methanol. Shorter chain fatty acid residues in the lipids have greater solubility in more polar solvents (Gunstone, 2004).

Fat and oil extraction can be done with single organic solvent or with organic solvent mixtures. Diethyl ether and petroleum ether are the most common single organic solvent used for extraction of lipids. Hexane is often used for extracting oils from raw material. Diethyl ether with a boiling point of 34^{o}C has better solvation ability for lipids compared with petroleum ether (Frank, 2004). Petroleum ether contains mainly pentanes and hexanes, is more hydrophobic and is therefore more selective for more hydrophobic lipids. Since most of the dietary lipids are TAGs, non polar solvents are used to extract and determine TAG content in foods. However often times a single nonpolar solvent may not extract the polar lipids from tissues, therefore to ensure complete and quantitative recovery of tissue lipids a solvent system composed of varying proportions of polar and non polar components may be used. Such a mixture extracts total lipids more exhaustively and the extract is suitable for further lipid characterization. Chloroform-methanol (2:1, v/v) solvent system provides an efficient medium for complete extraction of lipids from animal, plant or bacterial tissues (Akoh and Min, 2002). However due to the potential health hazards of chloroform, solvent mixtures containing alkane-alcohol-water mixtures such as hexane and isopropanol, with or without water have been successfully used to extract tissue and fish meal lipids. Hexane-isopropanol (3:2, v/v), heptanes-ethanol-water-sodium dodecylsufate (1:1:1, 0.05, v/v/v/w), methylene chloride-methanol (2:1, v/v), and hexane-acetone (1:1, v/v) systems are all various solvent combinations used to extract lipids from oilseeds as substitutes for hexane.

2.7.3 Soxhlet extraction

Several equipment and methods have been developed for lipid extraction using single solvent. Among them is soxhlet, which is a semicontinuous solvent extraction method. The Soxhlet extraction technique was invented in 1879 by Franz von Soxhlet for the determination of fat in milk. It has subsequently become the most used tool for solid–liquid extraction in many fields like environment, foodstuffs, and also pharmaceutics (Luque de Castro and Garcia-Ayuso, 1998).

Nowadays, Soxhlet apparatus is still common in laboratories and has been the standard and reference method for solid–liquid extraction in most cases. In soxhlet extraction the sample known weight is held in a filter paper thimble, the solvent accumulates in the extraction chamber for 5-10 min and then siphons back to the boiling flasks. This process continues for about 8 h with conventional soxhlet extraction (Luque de Castro and Garcia-Ayuso, 1998).

The soxhlet process involves an immersion period, washing period and recovery period. First the solvent is heated and during the immersion period the sample in the thimble is soaked in the boiling solvent for about an hour. This brings the sample into continuous contact with the solvent. This is then followed by a washing period where the thimble is removed from the boiling solvent and the solvent is allowed to drain back into the flask. Lastly there is solvent recovery which allows the solvent to be reused. The duration of extraction and temperature used depend on the sample and the type of solvent used. With soxhlet extraction, the sample is repeatedly brought into contact with the fresh portions of the solvent, thereby helping to displace the transfer equilibrium (Christie, 2007).

The temperature of the system remains relatively high since the heat applied to the distillation flask reaches the extraction cavity to some extent. No filtration is required after the leaching step. Sample throughput can be increased by simultaneous extraction in parallel, since the basic equipment is inexpensive. It is a very simple methodology which needs little specialized training; has the possibility to extract more sample mass than most of the latest methods (microwave-extraction, supercritical fluids, etc.), and is nonmatrix dependent (Clyde, 1996). There is a wide variety of official methods involving a sample preparation step based on Soxhlet extraction. Soxhlet extraction has certain disadvantages. The extraction requires a long period of time and a large amount of solvent is consumed. These solvents are very expensive and can cause environmental problems. Samples are usually extracted at the boiling point of the solvent for a long period of time and the possibility of thermal decomposition of the target compounds cannot be ignored.

Other disadvantages with solvent extraction are as followes: Solvent extraction requires the use of some toxic, expensive and harmful chemicals which can be left behind in the extracted oil.

Also the high processing temperatures required to boil the solvent can lead to a degradation of the extracted oil. Therefore the use of more environmentally and shorter extraction methods have been sought after.

2.7.4 Microwave extraction

In the last 10 years, there has been an increased interest in using techniques involving microwave-assisted extraction (MAE) and pressurised solvent extraction in analytical laboratories. The microwave dielectric heating effect uses the ability of some liquids and solids to transform electromagnetic energy into heat and thereby drive chemical reactions. Solid liquid system such as Soxhlet extraction has been used for many years. They are however time consuming and require a large amount of solvents (Virot, 2007). Microwave energy has been used as a heating source in analytical laboratories. The application of microwave energy as a heat source causes selective heating of the matrix over the extractant. The high localized temperature and pressure causes selective migration of target compounds from the material to the surroundings at a more rapid rate and with similar or better recovery compared with conventional extraction Ganzler *et al.,* (1986) were the first to report the use of microwave energy for the extraction of various types of compounds such as fat, pesticides and antinutritives from plant food and soil materials. Microwave assisted extraction reduces solvent consumption volumes. Also due to the short irradiation time prevents changes to compounds of interest (Pena *et al.,* 2006)

Microwave assisted extraction is a fast and efficient unconventional method developed for extracting analyte from solid matrixes.

Microwaves are electromagnetic radiations with a frequency from 0.3 to 300 GHz (Camel, 2001) microwaves possess electric and magnetic fields which are perpendicular to each other. The electric field causes heating via two simultaneous mechanisms, namely, dipolar rotation and ionic conduction (Thuery, 1992). Microwave heating effect arises from the interaction of the electric field component of the wave with charged particles in the material. Two effects can be used to explain the heating that arises from the interaction. A current will be induced which will travel in the phase with the field if the charged particles are free to travel through the material

(Kauffman and Christen, 2002). However, if the charged particles are bound within regions of the material, the electric field component will cause them to move until opposing forces balance the electric force. This results in a dipolar polarization in the material. Both conduction and dipolar polarization can lead to heating under microwave irradiation (Raghavan *et al*., 2005). When microwave energy is introduced to a dielectric material some part of the energy is transmitted, some part is reflected while some part is absorbed by the material which brings about heating of the material. Heating is due to molecular friction of dipoles in the material as they make an effort to reorient themselves with the rotation electrical field of the wave (Jassie *et al*., 1997). The power generated can be said to be proportional to the frequency of the source, the dielectric loss of the material and the square of the field strength within it.

The effect of microwave energy is strongly dependent on the nature of both the solvent and the solid matrix. Solvents generally used cover a wide range of polarities, from heptane to water. The type of solvent used depends largely on the compound of interest. Most of the time, the chosen solvent possesses a high dielectric constant and strongly absorbs microwave energy; however, the extracting selectivity and the ability of the medium to interact with microwaves can be modulated by using mixtures of solvents (Bassirro *et al.,* 1970). In some cases, the matrix itself interacts with microwaves while the surrounding solvent possesses a low dielectric constant and thus remains cold (Jassie *et al*., 1997). Localised heating leads to the expansion and rupture of cell walls and is followed by the liberation of essential oils into the solvent (Pare´ *et al*., 1997). This situation can also be obtained when a dry sample has been re-hydrated before extraction (Chen *et al.,* 1982). In fact, moisture content is essential in MAE because water locally superheats and promotes analytes to be liberated into the surrounding medium (Ma and Peltre, 1975). In addition, control of the water content of the matrix allows more reproducible results. Pare *et al.,* (1997), investigated the extraction of soy bean oil using microwave and reported that extracted oil yields increased with increasing length of microwave pretreatment and extraction time, because of the increased contact time between the solid phase and the liquid phase allowing for greater diffusion of the oil contained in the solid phase into the liquid phase.

2.5 ENZYMATIC HYDROLYSIS AND OIL EXTRACTION

Enzymatic hydrolysis has been applied to several processes such as preconditioning oilseeds and extraction of other bio-materials. This process has been used to extract oil from various oilseeds and oil materials such as soybean, sunflower and rape seed, to enhance oil extraction from these materials. Industrial processes for the extraction of edible oil from oilseeds involve a solvent extraction step, sometimes preceded by pressing. Often times the seeds are pressed and oil is further extracted from the seed cake using solvent extraction. Safety considerations in the use of organic solvents prompted attempts in the past to develop aqueous extraction but these were unsuccessful mainly due to the low oil yields (Rosenthal et al, 2001). It has however been reported that the low extraction yields of aqueous processes can be overcome by using enzymes that hydrolyse the structural polysaccharides forming the cell wall of oilseeds, or that hydrolyse the proteins which form the cell and lipid body membranes (Fullbrook, 1983; Lanzani et al, 1975; Sosulski *et al*., 1988). Enzymatic hydrolysis offers two advantages in oil extraction process: it gives higher yields of oil and higher quality of the meal, this field being considered as a new perspective of development in this kind of industry. The favourable effect of enzymatic pre-treatment on oil yield was first discovered in the 1950s. However, certain economic constraints such as high cost of enzymes did not allow its industrial application. In the 1970s this subject again attracted the attention of several researchers and the existing interest in this technology has led to the development of pilot and industrial processes (Domiguez et al, 1983).

Enzymatic pre-treatment of oil bearing materials before extraction can be applied to various oil extraction methods. The conventional oilseed processing involves the use of techniques such as flaking, cooking and crushing to rupture cell walls to make the oil more readily available during extraction (Owusu-Ansah, 1997). This however affects the quality of the oil and is more labour intensive. The use of enzyme as a more efficient way to rupture cell walls and liberate oil has thus been proposed.

2.5.1 Use of enzymes in oil extraction

Enzymes are used in various ways and combined with different oil extraction methods.
Due to the great amount of pressure applied, mechanical pressing leads to degradation and affects carbohydrates thus liberating oil; however the residue must not interact with the products to be purified. Enzymatic treatment offers a high yield and preservation of valuable extracted

components, because of the mild conditions employed (Olsen, 1988). In enzyme assisted pressing enzymes are used as a pre-treatment prior to pressing of the sample. The oil bearing materials are first flaked, enzymes are then added causing hydrolysis of the cell walls and making them more porous. This process breaks down the cellular structures causing degradation of the cells walls thus librating the oil (Owusu-Ansah, 1997).

Enzyme assisted pressing requires the use of temperatures lower than that which is employed for conventional pressing operation resulting in production of oil with a higher quality. Limitations however include high cost of enzyme and long incubation period required for certain oil bearing materials.

2.5.1.1 Enzyme assisted solvent extraction

Enzymes are also employed in solvent extraction of oil bearing materials such as oil seeds and fruits. Enzymes are added to the hydrated or flaked oil bearing material. When the desired reaction time is reached the flakes are dried to desired moisture content and oil is extracted using solvents. Enzyme assisted solvent extraction has been applied to increase oil recovery of oil seeds, fruits and legumes such as soybeans (Bhatnagar and Johri, 1987). The oil seed are soaked in the enzyme solution for a desired amount of time, dried and then followed by solvent extraction. Enzyme assisted solvent extraction offers certain advantages and this includes high oil recovery yield and a reduction in the amount of solvent used during extraction. However, long incubation periods, high cost of enzymes, and additional energy required for drying are the disadvantages of this method (Owusu-Ansah, 1997).

2.5.1.2 Enzyme assisted aqueous extraction

This involves addition of enzyme to the material that has been finely ground in water to make a paste. The extraction of oil from oil fruits is accomplished by the addition of hot water to the ground fruit, mixing of the paste and separation of the three phases, the solid, the aqueous and the oily. Enzymes are added to isolate oil from paste. The enzyme and paste mixture is then centrifuged to separate oil from an aqueous and solid phase (Lanzani *et al*, 1975) For enzyme

assisted extraction of seeds such as canola seed oil, the concentration of some undesirable compounds (glucosinolates, tannins, sinapine, and phytic acids) in the extracted meal is reduced. The limitation of this method is lower oil yields. About 18 to 25% of the available oil remains bound to the fine proteinaceous part upon final centrifugal clarification of the oil (Owusu-Ansah, 1997).

2.5.2 Research on enzymatic hydrolysis prior to oil extraction

Enzymatic hydrolysis procedures have been used by many researchers to achieve enhanced release of extractable oil from melon seed, sunflower seed, crushed soybean, cottonseed, castor seed, canola seed, soybrokens, soygrits and soyflakes (Bargale et al., 2000; Bhatnagar and Johri, 1987; Fullbrook, 1983; Kashyap *et al.*, 1997).

Most of these studies however were carried out on oil seeds and fruits. Shankar *et al.* (1997) reported an increase in the extractable oil of 0.3–2.3 percent from soybeans, soy splits, soybrokens, and soyflakes during enzymatic hydrolysis in combination with conventional pre-treatment unit operations. Sosulski *et al.,* (1993) also reported an increase in oil content from enzyme treated canola seeds. They reported an oil content in pressed cake from enzyme treated seeds of 17.4% compared to a cold pressed control of 16.8%. The oil quality was better than solvent extracted oil.

Bargale *et al.,* (2000) examined the effect of multi-enzymatic treatment before solvent extraction of soygrits, soyflakes, and expanded collets on the enhanced oil recovery. They demonstrated that proteases were more effective than cellulases or pectinases in enhancing oil recovery from soyflakes and expanded collets. They further reported that enzymatic pre-treatment along with high pressure and temperature could significantly increase the oil yield and oil extraction rate from ungroun soycollets. Kashyap *et al.,* (2007) in their study on oil extraction rates of enzymatically hydrolyzed soybeans reported that soyflakes hydrolyzed enzymatically under the optimal conditions had an oil yield of 24.9% as against 22.9% in unhydrolyzed soyflakes, showing an enhancement of 2.0% due to hydrolysis. Similarly, the oil yield for the enzymatically hydrolyzed soybrokens at the optimum conditions was 24.2% compared to 22.6% in raw unhydrolyzed soybrokens, showing an enhancement of 1.6% due to hydrolysis. The increase of

1.6–2.0 percent oil was due to the extra oil extracted from the meal as a result of the enzymatic hydrolysis since the total oil content of an oilseed is a fixed quantity. These results were in agreement with Domınguez, *et al.,* (1993), Smith *et al.* (1993), Shankar *et al.,* (1997), and Kashyap *et al*., (2006).

Further investigation has also demonstrated that enzymatic hydrolysis was useful in processing ground soybean and rapeseed to obtain better-quality oil and proteins of high nutritive value.

Rosenthal *et al.,* (2001) investigated the combined effect of operational variables and enzyme activity on aqueous enzymatic extraction of oil and protein from soybean and reported an oil yield increase from 41.8 to 58.7% of protease treated samples versus the control samples when heat treated flour was used, or when non-heat treated flour with large particle sizes was used in the extraction. Other enzymes used such as cellulase, hemicellulase and pectinase also reported an oil yield increase compared to the control. Protease however had the highest increase in oil yield. This effect allows not only the extraction of the nitrogenous compounds of proteinaceous origin, but also oil with an overall increase in the total solids extracted. Thus, the higher oil extraction in this article was explained by the solubilisation and hydrolysis of proteins, which possibly causes a breakdown in the protein network characteristic of the cotyledon cells cytoplasm, and in the protein (oleosin) based membranes that surround the lipid bodies, so liberating the oil (Bair, 1980; Huang, 1994; Murphy, 1993).

When enzymatically treated oilseeds are processed either by pressing or solvent extraction, the registered increase in oil is due to cell wall rupture. In oil fruits, the higher amount of oil recovered is due to this same reason, as well as to the disruption of the interphase of lipoproteic membranes (Adler-Nissen, 1986), thus allowing the dispersion of the colloidal system formed during grinding. This colloidal system presents a lipophilic phase in contact with oil and a hydrophilic phase in contact with water (Akoh and Min, 2008).

Studies by Rosenthal *et al*., (2001) showed that the conditions that favoured protein extraction (pH, temperature, particle size, agitation rate) also favoured oil extraction. This can be explained on the basis of the observation that lipid bodies enmeshed in the cytoplasm, which is

predominantly composed by protein, are themselves surrounded by proteinaceous membranes (mainly oleosins) (Bair, 1980; Huang, 1994; Murphy, 1993). In principle, it should therefore be possible to achieve an improvement in oil yield by using enzymes, especially, proteases. The use of certain proteases and cellulases has been reported to improve the yield of aqueous processes (Domiguez *et al*, 1993; Smith *et al.*, 1993; Yoon *et al.*, 1991).

2.5.3 Factors affecting enzymatic treatment

There are several factors affecting the enzymatic hydrolysis of oil bearing materials (such as oil seeds and fruits). Thus optimum condition must be established for the reaction. The variables can be adjusted during reaction time. This depends largely on the type of enzyme used.

2.5.3.1 Temperature

The temperature used should be in the range of maximum activity of the enzyme, such that the quality of the product will not be affected. Temperature used during treatments varies depending on the type of fruits or oilseeds being treated. Fullbrook (1983) used a temperature programme for rapeseed and soybean consisting of 60 min at 50°C, 120 min at 63°C and a short period (13 min) at 80°C to inactivate enzymes. Lanzani *et al.*, (1975) treated sunflower, rapeseed and peanut using a sequence of increasing temperatures (40°C, 50°C and 65°C) during 3 h. This is to show that temperature ranges and duration of treatments vary for different samples (Dominguez et al, 1994).

2.5.3.2 pH

The pH used should also be that which is optimal for the enzyme activity. The pH range of 4-5-5.5 is generally suitable; 3-8 is the range of maximum activity (Dominguez *et al*., 1994).. Generally the pH used depends on the enzyme.

2.5.3.3 Dilution ratio

The amount of water added during the treatment or used to prepare the enzyme solution can also have a great effect on the recovered oil yield. This also varies and depends on the type of fruit or oilseed. Buenrostro and Lopez-Monguia, (1986) have found that the maximum yield of avocado oil after pressing was reached with dilution ratios of 1/5, while with coconut the best yields were obtained at a 1/4 dilution ratio. This effect has also been studied for the extraction from olive pastes, after drying the fruit to a final moisture content of 10% (weight) and addition of the enzymatic solution in buffered media. Enzymes showed their maximum activity in media with a water content of 35% or more (Dominguez et al, 1994).

2.5.3.4 Enzyme concentration

An increase in the enzyme concentration is known to increase the rate at which the oil is separated in an aqueous system, the optimum concentration must however be established. Montedoro (1986) reported that cellulase enhances the oil extraction at 25-30 g/100 g olive, its efficiency being considerably reduced when working at higher or lower concentrations. Acid protease yielded the maximum at 50g/100g, they however stated that hemicellulase did not present a marked optimum and the better values range between 50 and 80 g of enzyme/100 g of paste. Mixtures of enzymes yield more oil due to their combined effect on colloidal and lipoproteic structures. Mixture of enzyme activities provides higher extraction oil yields (Montedoro *et al.,* 1974).

The influence of different formulations on olive oil yield obtained by single pressing after enzymatic treatment showed that mixed cellulase associated with pectinase, or cellulase with acid protease was more efficient in increasing the yield than cellulose. An increase in the enzyme concentration makes the emulsion more unstable. Therefore optimum enzyme concentration should be established for effective and efficient enzyme treatment (Dominguez *et al.,* 1993).

2.5.3.5 Reaction time

Enzyme incubation times reported in the literature (0.33-2 h) were enough to achieve significant increase of oil yield. The enzymatic incubation is maintained during the mixing stage substituting the addition of hot water in the conventional process (Deng *et al.,* 1992). Studies conducted on oil seeds reported that incubation time of 90 min is significantly more favourable than shorter ones and the amount of oil obtained is not increased futher by longer times of contact with enzymes. This however depends on the type `of oil seeds because of the differences in their cell wall composition (Dominguez et al, 1994).

2.6 PEF (Pulsed Electric Field)

Pulsed electric field (PEF) technology consists in the application of short duration pulses (microseconds) of high electric field strengths (0.1 to 50 kV/cm) to products placed between 2 electrodes. Short-duration high-intensity field strengths cause electroporation of cells and an increase in their permeability (Zimmerman *et al.,* 1986). In recent years, the consumer demand for high-quality natural foods has increased. Pulsed electric field (PEF) is a nonthermal food processing technology, which has received increased interest during the last years. The application of PEF has been proposed as an alternative to the conventional method of pasteurisation. Due to a number of advantages in comparison with thermal treatments it has gained increasing interest. The use of pulsed electric field is based on the knowledge that an external electric field can induce critical electrical potential across the cell membrane (Angersbach *et al.,* 2002). This leads to electrical breakdown and local structural changes of cell membranes, which increases the permeability of the cells.

The application of an external electrical field results in an electric potential across the membranes of biological cells. PEF treatment of food commodities involves the use of different

variables. This includes treatment time, pulse form and pulse energy, electric field strength and temperature. This effect can also be used for non-thermal inactivation of microorganisms. When the potential difference reaches a critical value, called the breakdown potential, localized electrical breakdown of the membrane occurs and cell permeability increases (Zimmerman *et al.*, 1974). Structural changes of the cell membrane, such as reversible and irreversible formation of pores, occur depending on the intensity of the electrical field and other variables.

Until now, there is not much known about the impact of pulsed electric fields on the recovery of oils from oilseeds and the impact on high fatty plant cells. Few authors have reported good results on the use of PEF in enhancing oil yield; however no work has been done to investigate the use of PEF in enhancing oil from animal tissues, which is one of the objectives of this research work.

2.6.1 Previous work on application of PEF

Guderjan *et al* (2005) investigated the impact of pulsed electric field treatment on the recovery and quality of plant oils using maize, olives and soybeans. In maize samples, they reported that the dry milled germs with a moisture content of 66.7% had a disintegration index of 29.7% at a field strength of 7.3 kV/cm compared with hand separated germs with a moisture content of 33.2% and a disintegration index of 8.9%. They also stated that after PEF, a phytosterol yield up to 1039.4 mg/100 g oil and oil yield up to 43.7% were acheived, which means a maximum increase of 32.4% of phytosterols and 88.4% of germ oil in comparison to the untreated samples. For soybean samples they reported that a maximum amount of daidzain of 288 Ig/g was obtained with 50 pulses at an energy field of 1.3 $kVcm^{-1}$ and an energy impact of 1.857 kJ/kg, which implies an increase of 20% in comparison to the reference.

Guderjan *et al,* (2007) also conducted a study on the application of pulsed electric field in the production of oil and functional food ingredients of rapeseed oil. Hulled and non-hulled rapeseeds were used, and the effect of pulsed electric field on different oil separation methods was investigated. Soxhlet extraction was used to quantify the oil yields of both untreated and permeabilised rapeseed. They reported that oil yield of hulled and non-hulled rapeseed increased

by the application of pulsed electric fields. At electrical field strengths higher than 1 kV cm^{-1}, permanent pores were generated, which resulted in improved mass transfer and enhanced extraction of oil and plant ingredients. At 7.0kVcm^{-1} and 120 pulses, oil yield of hulled rapeseed increased from 23% up to 32%; and for non-hulled rapeseed from 43% up to 45%. The effect of PEF was more distinctive for hulled rapeseed. Oil separation was however incomplete and pressing followed by hexane extraction recovered an oil yield between 40% and 51%. They concluded that PEF could be used as a pre-treatment for cell disruption before oil separation. It is known that different extraction methods of the oil affect the composition and concentration of minor components of oil. Their data also indicate that pulsed electric fields have an effect on improving oil quality.

Several studies have been done to investigate the effect of PEF on various other compounds. In the case of betanine extraction from beetroot by using PEF, the literature shows very promising results as reported by Charlermchat *et al.,* (2004); Fincan *et al.,* (2004). Their studies were however carried out using field strengths less than 1 kV/cm; it is believed that the potential of PEF for betanine extraction could be even greater at higher field strengths, or when different extraction conditions are used.

Lebovka *et al.*, (2004); Fincan *et al.*, (2002); Jemai *et al.*, (2006) have all reported on the disintegration of cell membranes in plant tissue by the application of PEF. These researchers investigated the extraction efficiency of fruit juices with PEF as a pre-treatment before mechanical pressing or solvent extraction. The application of PEF was focused mainly on its effect in increasing extraction properties, as well as investigating it effect in the inactivation of micro organisms in fruit juices. Chalermchat *et al.,* (2005) as well as Fincan *et al.,* (2004) demonstrated an influence of PEF intensity on solid–liquid expression from different plant tissues. It has also been shown that pulsed electric fields can be used to inactivate micro-organisms in juices with subsequently very good sensory properties (Min *et al.*, 2003).

The application of PEF as a pre-treatment before extraction, e.g., of sugar from sugar beet slices, produces juices of higher quality. The quality of cellular juice obtained after PEF treatment was said to be higher than juice obtained after thermal pre-treatment (El-Belghiti *et al.,* 2005). A

good pressing efficiency as well as high juice purity and yield at a minimum power consumption of approximately 5–20 kJ kg^{-1} has also been reported (Knorr and Angersbach 1998).

Application of PEF for enhancing the pressing and diffusion extraction has a number of potential benefits. It does not require introduction of enzymes which are very expensive. It is very useful in preserving organoleptic characteristics, flavouring agents, vitamins and nutritive values of juices as compared with conventional thermal pasteurization treatment (Ade-Omowaye et al, 2003). PEF treatment is often accompanied by a stream of natural solutes leaking from the tissue and results in a considerable enhancement of the diffusion (Eshtiagi *et al.,,* 2002). It allows to reduce the diffusion time and to decrease the water consumption in the washing treatment procedure. As a supplementary benefit, the PEF treatment provides with a possibility of microbiological and enzymatic inactivation of the released products (Ade-Omowaye et al, 2003).

2.8 BIO-DIESEL

Due to the increase in the price of the petroleum and the environmental concerns about pollution from automobile operations, biodiesel is becoming a developing area of high importance. Alternative fuel development is important for securing the supply of future transportation fuels, as well as for cleaner fuel utilization. Among the alternative and renewable fuel sources being considered are various types of fats and oils derived from plant and animal sources.

Bio-diesel is defined as the mono alkyl esters of long chain fatty acids derived from renewable feed stock, such as vegetable oil or animal fats, for use in compression ignition engines (Sharma et al, 2008). Biodiesel, considered as a possible substitute of conventional diesel fuel is commonly composed of fatty acid methyl/alkyl esters, obtained from triglycerides by transesterification with methanol/or other alcohols. Biodiesel is biodegradable and nontoxic, has low emission profiles and so is environmentally beneficial (Krawczyk, 1996). Various oils have been used in different countries as raw materials for biodiesel production owing to their availability. Soybean oil is commonly used in United States and rapeseed oil is used in many european countries for biodiesel production, whereas, coconut oil and palm oils are used in Malaysia for biodiesel production (Sarin *et al,* 2007). Transesterification of edible oils has also

been carried out from the oil of canola and sunflower. Other edible and non-edible oils, animal fats, algae and waste cooking oils have also been investigated by researchers for the development of biodiesel (Wang *et al.,* 2007)

.

The use of vegetable oils as sources of diesel would require more efforts to increase the production of oil seed and to develop new and more productive plant species with high yield of oil. Vegetable oils occupy a prominent position in the development of alternative fuels although there have been many problems associated with using it directly in diesel engine. Sharma *et al.,* (2008) highlighted a few of the problems. These include:

1. High viscosity of vegetable oil interferes with the injection process and leads to poor fuel atomization,
2. The inefficient mixing of oil with air contributes to incomplete combustion, leading to high smoke emission,
3. The high flash point attributes to lower volatility characteristics,
4. Lube oil dilution,
5. High carbon deposits,
6. Ring sticking,
7. Scuffing of the engine liner,
8. Injection nozzle failure,
9. Types and grade of oil and local climatic conditions, and
10. Both cloud and pour points are significantly higher than that of diesel fuel,

However the production of methyl esters from oils overcomes these problems,

The major constraint of direct application of waste frying oil lies in its higher amount of free fatty acid. The free fatty acid level of fresh soybean oil has been reported to change from 0.04% to 1.51% after 70 h of frying at 189.85^{o}C. Hence, due to higher free fatty acid, direct conversion of waste restaurant oil and animal fats to biodiesel via alkaline catalyst is not possible. The level of free fatty acid, therefore, is reduced by an acid catalyst.

Following this advantage, researchers have advocated the use of rendered animal fats as biodiesel feedstock (Adebanjo *et al*, 2005). The use of animal fat instead of vegetable oil to produce alternative diesel is an effective way to reduce the raw material cost because its cost is estimated to be about half the price of virgin vegetable oil. Because meats cannot be produced without the simultaneous production of waste fat, a large amount about 27.9 million metric tons per year globally of animal waste fat is unavoidably produced in the process of supplying meat (Adebanjo *et al*, 2005). Also, due to the recent Bovine Spongiform Encephalopathy (BSE) crisis, the use of animal-derived products to feed cattle is now severely restricted. Therefore, using animal fat as fuel could also help to solve the problem of waste disposal (Leung and Guo, 2006). Considerable research has been done on the use of vegetable oils as diesel fuel. That research includes restaurant oil waste and waste cooking oil (Canakci, 2007; Wang *et al.*, 2007). Animal fats, although mentioned frequently, have not been studied to the same extent as vegetable oils.

2.9 ANALYSIS OF LIPIDS

Lipid analysis is usually required to determine the composition and structure of the lipid extracted from the sample. The characterization provides information on the caloric value, nutritional quality and safety of the lipids.

2.9.1 Fatty acid profile

Fatty acids may be found in scarce amounts in free form but, in general they are combined in more complex molecules through ester or amide bonds (Akoh and Min, 2002). The presence of free fatty acid in oil is an indication of insufficient processing, lipase activity or other hydrolytic actions. Free fatty acids of oils can be determined colorimetrically by dissolving oil in chloroform (or benzene), then allowing the free fatty acids (FFA) to react with a cupric acetate solution. The organic solvent turns to a blue color due to the free fatty acid–cupric ion complex, which has a maximum absorbance between 640 and 690 nm Fourier transform infra red (FTIR) spectroscopy can also be used to determine the content of free fatty acids (Akoh and Min, 2002).

Gas chromatography is the most frequently used technique for analysis of the fatty acids profile of fats and oils and normally requires methyl esterification of fatty acid bound on the tag molecule. The preparation of fatty acid methy esters (FAME) requires fat extraction from biological materials with organic solvents, followed by transesterification of the fat to form fatty acid methyl esters (Subramaniam et al, 2003). The generation of methyl esters can be done in acidic or in alkaline conditions on isolated lipids or fatty acids but also directly by a one-step procedure combining lipid extraction and transesterification on small amounts of dried tissue (Hui, 2006).

For the quantification of individual fatty acids in any acylated lipids, gas liquid chromatography (GLC) must be adopted. Mass spectrometry coupled to GC is the most powerful tool for identifying fatty acids separated by GLC. IR spectroscopy and Raman spectroscopy sometimes used to detect trans-fatty acids and isomers of cis-unsaturated fatty acids (Caballero et al, 2003).

2.9.2 Oxidation of lipids

This is a reaction of an oil or fat with oxygen.
Several enzymes present in animal tissues, such as lipoxygenases, catalyses oxidation reactions (Hui, 2006). Unsaturated fatty acids, especially di- and triunsaturated acids, are more easily converted into free radicals.

The analysis of lipid oxidation products is difficult because the lipid oxidation reactions are consecutive but at the same type overlapping. Therefore, the analytical methods used should be selected and/or adapted to the composition and amount of lipid oxidation products. The best procedure is to combine several methods based on different principles, e.g. chemical, chromatographic, and spectrometric methods that can measure both primary and secondary oxidation products (Afaf and Jan, 2005).

Measurement of the degree of oxidation is usually performed by measuring the changes in the peroxide value (PV) of a given weight of fat with time (Harry, 1995). Iodine is liberated by hydroperoxides in the oil in the presence of excess iodide in a stoichiometric ratio. The amount of iodine present is determined by titration with a standard sodium thiosulfate solution using astarch indicator, thereby reflecting how much peroxide is present in the oil or lipid extract.
Another approach for the determination of peroxide value is a spectrophotometric method based on the ability of peroxides to oxidize iron (I1) to iron (II1). The ferric ion forms a complex with xylenol orange (FOX), whose concentration can be determined spectrophotometrically (Ronald, 2005).

2.9.3 Hydrolysis of lipids

Water, introduced into the fat from the food, causes hydrolysis of the ester bonds in the fat, producing diglycerides, monoglycerides, free fatty acids and (Clyde, 1996). In some situations this partial hydrolysis yielding some monoglycerides and diglycerides will eventually be carried to completion, resulting in glycerol and free fatty acids (Harry, 1995). Hydrolysis is accelerated by high temperature and pressures and an excessive amount of water. Lipids may be hydrolyzed with immobilized lipases too.

Complex lipids, those that contain esterified fatty acids, can often be best analyzed by hydrolyzing the esterified fatty acids, and quantitatively analyzing them via GC. Hydrolysis is usually carried out under mild alkaline conditions (0.5 to 1.5 KOH in methanol). After hydrolysis, the mixture is usually acidified to protonate the fatty acids; then they are extracted (and separated from glycerol, salts, and other nonlipid components) by adding hexane or employing other lipid extraction methods. GC methods for fatty acids analysis usually require the conversion of free fatty acids to fatty acid methyl esters (FAME). Several hydrolysis-methylation and transesterification methods are commonly used to prepare fatty acid methyl esters (Semih, 2005).

2.9.4 Degree of unsaturation

The degree of unsaturation can be expressed in terms of the iodine value of the fat. Iodine value is defined as the number of grams of iodine (or other halogen) that will react with the double bonds in 100 g of fat. The reaction occurs with both cis and trans double bonds. The higher the iodine value, the greater the unsaturation of a specific oil or fat (Harry, 1995)

Hydrogenation is used to measure the degree of unsaturation of acetylenic or conjugated double bonds. Such fats do not absorb halogen readily; however, the addition of hydrogen to them is considered to be quantitative. This method is essentially a catalytic reaction of heated lipid; the amount of hydrogen absorbed is determined under standard conditions. The results are expressed on a mole basis or on the basis of IV (Akoh and Min, 2002).

At the low frequency end of the fingerprint region of IR (1500–900 cm^{-1}), a band due to the CH=CH bending absorption of isolated trans double bonds would be observed. Beyond the isolated trans bond is another group of CH absorption, in this case bending vibrations, including a very strong cis absorption. The combination of cis and trans absorption provides a measure of the total unsaturation or iodine value (Afaf and Jan, 2005).

Chapter 3: Enzymatic pre-treatment and microwave assisted extraction of oil from animal discards

Enzymatic pre-treatment and microwave assisted extraction of oil from animal discards

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ABSTRACT

An investigation was conducted into the use of protease enzyme pre-treatment and microwave assistance to improve the efficiency of hexane extraction of pork and chicken fat obtained from processing plants. The conditions for optimizing both enzyme pretreatment and microwave assisted extraction were evaluated by varying the incubation times (30min, 1h, 2h, 3h) with papain and the duration of exposure to microwave irradiation (5min, 7.5min, 10, 12.5min), which was applied in a stepwise manner (on-off- on). Soxhlet extraction was used as the standard method for assessing the efficacy of the microwave assisted procedure. Fat yield was determined gravimetrically, and the extracted fat was analysed for hydroperoxide value and p-anisidine values as indices of the extent of oxidation. Gas chromatography was used to determine the fatty acid composition of the fat. Pre-treatment with enzyme for 2 h of incubation and microwave irradiation for a total 15 min resulted in the highest fat yield. The oil extracted by enzyme assisted microwave extraction for 15 min were comparable in terms of yield and fatty acid composition to that obtained using soxhlet.

Keywords: Enzymatic pre-treatment, microwave extraction, chicken, pork

3.1 Introduction

Large quantities of animal discards are produced from the slaughtering and processing of livestock, and much of this waste biomass is animal fat. According to Adebanjo *et al.,* (2005) about 27.9 million metric tonnes of animal fats are produced globally each year, and these animal discards can be used to produce biodiesel (Dominguez *et al,*1993). Oil crops such as soybean and canola can also be used for biodiesel production (Canacki and Gerpen, 2003), but the use of edible crops for biofuel production is viewed as contributing to high food prices and accentuating the global food crisis (Adebanjo *et al*, 2005). The use of animal discards to produce biodiesel not only avoids the food-fuel dilemma, but reduces the problem of animal waste disposal and contributes to the global initiatives to increase the global supply of biofuels (Pearl, 2002).

Fat extraction is a critical step in the production of biodiesel but there is a scarcity of research exploring various conditions for optimization fat extraction from animal discards. Enzymatic hydrolysis has been used as a pretreatment to enhance oil extractability from oil seeds such as sunflower (Dominguez *et al,* 1993) and soybean (Kashyap et al, 1997, Rosenthal *et al*., 2001); but economic constraints such as high cost of enzyme precluded industrial application (Domı´nguez *et al.,* (1993). Enzymatic hydrolysis as a pretreatment prior to solvent extraction has been shown to increase oil yield by 1.6 to 2.3% by Smith *et al*., (1993). Enzymatic hydrolysis is thought to offer two advantages in oil extraction process; it results in greater yields and better quality oil when compared to samples extracted by mechanical pressing. The higher yield can be attributed to action of the enzyme on the cell walls. The enzyme helps in breaking the cell walls liberating oil (Kashyap *et al*, 1997).

Microwaves are electromagnetic waves with frequencies ranging between 300MHz to 30GHz which have been studied as a means of assisting the extraction of various compounds from plant and animal tissues and this technology has the potential to increase the yield of fat from the biological tissues (Ganzler *et al,* 1986). Microwave energy brings about selective heating of the sample over the solvent and thus leads to the desired compound from the sample matrix into the extractant (Pare *et al*., 1997). Ganzler *et al*., (1986) was the first to use microwave to extract

crude fat from food matrix using organic solvent; the authors observed that fat yield achieved with 3.5 min of microwave irradiation was comparable to that obtained with 8 h of conventional soxhlet extraction. Compared to conventional extraction, microwave assisted extraction consumes minimal amount of solvent, reduces by-product formation and consumes less energy; this makes it highly efficient and economical method of fat extraction.

Optimization of extraction of compounds such as free amino acids (Agnes *et al.,* 1998), asthanxanthin (Zhao *et al.,* 2006) has been well investigated, but there is a paucity of information in the literature on the optimization of the extraction of fats and oils using microwave. The aim of this study was to investigate the use of enzymatic hydrolysis to enhance oil extraction from chicken and pork processing discard for the production of biodisel.

3.2 Material and Methods

3.2.1 Source and preparation of Samples

The chicken fat discards were obtained from a chicken farm in Quebec and pork fat samples were obtained from a local supermarket in Quebec. Non fatty tissues such as bone were hand removed. In order to obtain a homogeneous sample and minimize sampling error, the source material was minced in a waring blender.

3.2.2 Enzyme pre-treatment

60 g of the minced tissue was weighed into a beaker and treated with 0.06 g of papain dissolved in 15ml of phosphate buffer pH 7.0. The homogenate thus formed was incubated in a water bath at 45^{o}C for various time intervals: 0, 30 min, 1 h, 2 h, and 3 h. After incubation, the samples were rapidly frozen in liquid nitrogen and freeze dried; the dried samples were weighed for microwave assisted extraction.

Oil was extracted from the protease treated samples and non protease treated samples using the soxhlet extractor. The solvent was evaporated using nitrogen after which the oil were placed in an air tight sealed glass bottles labelled and stored in the freezer at -53^{0}C for further analysis.

3.2.3 Extraction Methods

3.2.3.1 Microwave extraction

About 2.00 g of freeze dried samples were weighed and then transferred into 250 ml quartz extraction vessel. 30ml of hexan was then added to the vessel.The vessel was inserted in the extraction cavity, fitted with a condenser and irradiated in the following sequence at full power (600 W):60 s on, 30s off, and 90 s on. Microwave system 2 (CEM Corporation, North Carolina, USA). The temperature of the extractant was taken before and after irradiation. After irradiation, the glass vessel was washed with 10 ml of hexane and the washes were collected in the extraction vessel. The extract was filtered under vacuum through a Whatman filter cup and collected in a round bottom flask. The weight of the flask was recorded. The residue in the

extraction vessel was washed with 20 ml hexane and the wash was filtered and added to the extract. The combined extracts in the round bottom flask were evaporated under vacuum in a rotary evaporator Buchi R115). After the solvent had evaporated, the flask was allowed to cool for 30min to room temperature and then reweighed with the oil. The yield of fat was calculated as follows:

$$\% \text{ fat yield} = \frac{\text{Weight of fat}}{\text{Weight of sample}} \times 100 \quad \text{..... 3.1}$$

Fat extractions were performed in triplicate.

Mode optimization

To optimize the fat yield using microwave, the above step was repeated using the following modes to determine which mode achieved the best oil yield.

Mode I (60 on, 30 off, 90 on)

Mode II (120 on, 60 off, 180 on)

Mode III (180 on, 90 off, 270 on)

Mode IV (240 on, 120 off, 360 on)

The following samples were analysed; control (no protease treatment), 30 min protease treatment, 1 h protease treatment, 2 h protease treatment and 3 h protease treatment.

3.2.3.2 Soxhlet extraction

The protease treated and non treated samples were all subjected to soxhlet extraction. All samples (5-10grams) weighed into thimbles and the extraction glass vessels were weighed; 60 ml of hexane was the added to the extraction glass vessels. The soxhlet extraction program was set at Immersion 1 h, rinsing 1 h followed by evaporation 45 min. The boiling temperature of the solvent was set at 70^{o}C. After soxhlet extraction the glass vessels were placed in an oven at 300^{o}C for 30 min to complete solvent evaporation. The extraction glass vessels were then cooled in a desiccator and re-weighed. The yield (%) of fat was calculated as follows equation 3.1

3.2.4 Analytical Methods

3.2.4.1 Hydroperoxide determination

The modified Xylenol orange to evaluate formation of lipid hydroperoxide method proposed by Eymard and Genot (2003) was used.

Preparation of ferrous oxidation reagent (FOX2)

2.5mM Ammonium ferrous sulphate was dissolved in 250mM H_2SO_4. 25ml of this solution was then mixed with 19mg of xylenol orange previously dissolved in 50ml of methanol. The volume of the mixture was finally completed to 250ml with methanol. Concentrations of xylenol orange, ammonium ferrous sulphate, and H_2SO_4 were 100µM, 0.25mM, and 25mM, respectively. FOX2 reagent was prepared just before use.

Reaction conditions and absorbance reading

25µl of animal oil were pipetted into microtubes (1.5 micro locking tubes,) and made up to 150µl with methanol. 1350µl of FOX2 reagent were added. Final concentrations of ferrous ammonium sulphate, H_2SO_4, and xylenol orange in the reaction mixture were 0.22mM, 22.5mM and 90µM respectively in water/methanol (10/90;v/v). The reaction mixtures were mixed and then incubated in darkness at room temperature for 50min. Their absorbances were read at 560nm against a blank (150µl methanol and 1350µl FOX2 reagent).

3.2.4.2 Measurement of *p* -Anisidine Value

The *p* -Anisidine value was measured using the AOCS official method Cd 18-90 as follows:
The oil sample was weighed (0.5-4.0g) into a 25 ml volumetric flask. The oil sample was dissolved and diluted to known volume with isooctane. The absorbance of the solution was measured at 350 nm with isooctane as blank. Next, 5 ml of the fat solution was pipetted into a 10 ml Teflon lined screw capped test tube while exactly 5 ml of the solvent (isooctane) were pipetted into a second test tube. Then 1 ml of the *p*-anisidine solution was added to each test tube

and shaken. After 10 min the absorbance of the solution in the first test tube was measured using the solution in the second test tube as blank.

3.2.4.3 Fatty Acid Composition

Fatty acid methyl esters (FAME) were prepared using sodium methylate as catalyst according to Fournier *et al.*,(2006). About 20mg of the oil sample was weighed into a 30 ml Teflon lined screw capped test tube. Next, 1 ml of toluene and 2 ml of a 0.5 M sodium methylate solution in methanol were added. The solution in the test tube was then incubated in a water bath at 50°C for exactly 5 min. After cooling, 100 µL of glacial acetic acid was added using a glass syringe, followed by 5 ml of hexane and 5 mL of distilled water. The test tube maintained at 4^0C was centrifuged for 3 min at 2000 rpm. The hexanic phase containing fatty acid methyl esters was pipetted into glass bottles with Teflon lined screw caps. The aqueous phase is extracted at 4^0C a second time with 5 ml of hexane centrifuged for 3 min at 2000 rpm. The two hexanic layers were put together then dried under a flow of nitrogen. The fatty acid methyl esters were then diluted to a concentration of 0.1 mg/ml with hexane.

The FAME were analysed using a Hewlett-Packard Model 5890 capillary gas chromatograph (Palo Alto, CA, USA) with a DB-23 60m 0.25 ID 0.15 micrometer film thicknesses, FID (Flame ionized detector) was used.

3.2.5 Statistical analysis

Data were analyzed using SAS version 9.13 for windows (SAS Institute, Cary, NC, USA). The data were tested for normality. Data was considered normal if it had a P value greater than 0.05 according to the Shapiro-Wilk statistics. Analysis of variance (ANOVA, SAS PROC GLM) was used to analyze the normally distributed data. All results were reported at a 0.05 significance level.

3.3 Results and Discussion

3.3.1 Optimization of enzymatic treatment

The optimum enzymatic hydrolysis conditions based on maximum oil yield from the pork and chicken samples using 0.1% v/w enzyme pre-treatment for 30 min, 1 h, 2 h and 3 h incubation periods were investigated and the fat yield of the sample from the various incubation periods were determined using both soxhlet and microwave extraction.

3.3.2 Oil extraction from enzyme pre-treated samples

The data for the enzymatic hydrolysis pre-treatment are presented in figure 3.1, based on incubation period and fat yield obtained. This was carried out to find out which of the incubation period will give and highest fat yield. Figure 3.1, shows that enzyme pretreatment increased fat yield compared to non hydrolyzed samples. The fat yields for control samples were 63.1% and 61.6% for the chicken and pork samples respectively. Pre-treatment of the chicken samples for 30 min, 1 h, and 2 h resulted in fat yields of 64.5%, 77.4% and 81.7% respectively. Based on this data it is clear that a fat yield increase of about 21.7% (P=0.0003) was obtained when the chicken samples were pre-treated with the protease for 2 h.

For the pork samples, a similar trend was observed. Enzyme pre-treatment for 30 min, 1 h and 2 h resulted in increased fat yield of 71.2%, 76.4% and 78.2% respectively. A fat yield increase of about 16.6% (P=0.0179). There was an overall significant difference in chicken samples (P=0.0001) and in pork samples (P=0.0141), also there was a significant difference between control and enzyme pretreated sample; 1h (P=0.0022), 2h (P=0.0003), 3h (P=0.0131) indicating that this pretreatment were more effective on improving fat yield. There were no significant difference between control samples and sample pretreated for 30 min (P=1.000). The difference in fat yield can be attributed to the effect of the enzyme hydrolysis, breaking up protein molecules associated with the fat and oil, making it more available for extraction as rationalized by (Kashyap *et al*., 1997).

In both the pork and chicken samples, a decrease in fat yield was observed when enzyme pre treatment was extended for 3 h, the statistical analysis showed that there are no significant difference between oil yield of samples pretreated for 2h and samples pretreated for 3h (P=0.2178). This might be due to the prolonged exposure time resulting in autolysis by endogenous lipases and/or degradation of the fat via oxidation. From these results it was concluded that incubating the sample with 0.1% enzyme concentration at 45^{o}C for 2 h gave the highest fat yield.

3.3.3 Optimization of microwave assisted process oil extraction

We optimized the microwave program for better fat yield. Increasing exposure programs were used. The data for the microwave assisted extraction are summarized in Figure 3.3 for chicken and Figure 3.4 for pork. Using the microwave in extraction mode of 60 s on 30 s off 90 s on gave an average fat yield of 76.42% for pork samples 1 h enzyme pre-treated compared with 78.23% fat yield for 2h enzyme pre treated pork samples. The same microwave extraction mode when applied for the chicken samples yielded 64.54% versus 81.7% from the 1 h and the 2 h enzyme pretreated samples respectively. The microwave extraction mode was increased in steps of 60 s on 30 s off and 90 s on until a plateau was reached. As shown in Figure 3.4, the plateau for the fat yield was attained after irradiating at a sequence of 180s on, 90s off, 270 s on total irradiation time of 7.5min. This sequence gave the maximum oil yield of 86.33% and 91.09% in chicken samples hydrolysed for 1hr and 2hrs respectively and 87.19% and 91.86% in the corresponding pork samples. There were no significant difference between microwave sequence 180s on, 90s off, 270 s on and subsequent sequences (240 s on 120 s off 360 s, 300 s on 150 s off 450s on) (P=1.000).

The temperature of the extractant (hexane) used has a great effect on the efficiency of the microwave extraction. The boiling temperature of hexane is 69^{o}C (Pare et al., 1997). For the sequence (180s on, 90 s off, 270 s on) that gave the best yield the temperature of the solvent before irradiation was 35^{o}C and after irradiation it was 68^{o}C. The difference in temperature before and after irradiation indicated that localized heating of the sample has taken place (Virot

et al., 2008). The heating caused the migration of some molecules from the sample to the surrounding solvent, thus increasing the solvent's temperature. No solvent was lost because the extraction vessel was fitted with a condenser during irradiation. Microwave solvent extraction of fats and oils may occur by one of these two proposed extraction mechanisms or as a combination of both: (a) for a sample with a high dielectric loss, efficient extraction can be performed using only microwaves without any added solvent. This is possible since the water inside the sample matrix will be locally heated. Microwaves interact with the free water molecules present in the glands and vascular systems. Thus, such systems undergo a dramatic expansion, with subsequent rupture of the tissue, allowing the oil to flow towards the damaged layer. (Virot et al., 2007)

3. 3.4 Comparison with soxhlet extraction

Table 3.1 presents a comparison of soxhlet extraction versus microwave extraction of fat from the enzyme pre treated samples that gave the best yield; microwave program sequence took 7.5min, while soxhlet extraction lasted for 3hrs (45 min immersion time, 1hr washing time and 45 min recovery time). Fat yield difference between soxhlet and microwave is more than 6% in both chicken and pork samples. As shown in Table 1 there are significant differences between fat yields obtained from microwave extraction and soxhlet extraction ($P < 0.05$). This shows that microwave extraction for 7.5 min is as efficient as soxhlet extraction for 2.5 h. The experiments also showed that enzymatic hydrolysis was more effective in improving fat yield using microwave assisted extraction.

3.3.5 Oxidation Assesment

Lipid oxidation is a major cause of deteroriation in fats and oils. It starts with the production of primary oxidation products such as free radicals and hydroperoxides. Lipid oxidation was measured by assessment of primary (peroxide value) and secondary (p-anisidine value) oxidation compounds (Aubourg *et al.*, 1997). Table 3.2 presents the results of the oxidation assessment. It can be seen that the hydroperoxide value in the enzyme pretreatment increases with increased time of incubation for chicken samples. The hydroperoxide value for enzymatically hydrolysed

pork samples when incubated for 30min is 7.66 mM OOH/kg and reduced significantly to 4.78mM OOH/kg after 2 hours of incubation.

The high hydroperoxide value obtained with low incubation period can be due to the fact that raw samples obtained from the butcher were highly oxidized at the outset. With increase incubation period the hydroperoxide values were seen to reduce, this is because the oil samples have started producing secondary oxidation products from the primary oxidation products. This can be confirmed from the p-anisidine value as shown in Table 3.2. The p-anisidine value obtained at 30 min was lower (21.35%) than that obtain (59.65 %) after 2 h incubation in the chicken samples. This shows that secondary oxidation products such as aldehyde and ketones are highly present in the samples after prolonged enzymatic treatments. The temperature used during incubation could also have an effect on these oxidation products. Similar trends was observed for both samples however it can be seen that the chicken samples showed higher hydroperoxide values compared to pork, this is because chicken fat are more saturated than pork fat and therefore more prone to oxidation.

3.3.6 Fatty acid composition

Fatty acids composing the oil extracted from both chicken and pork samples were converted into FAME derivatives and analyzed by GC. The methyl esters were identified and the relative percentages in peak area of the compounds are presented. Table 3.3 presents the major fatty acids identified for the oil extracted from enzyme pretreated chicken samples and control samples for comparison. As seen in Table 3.3 Methyl palmitate, methyl oleate and methyl linoleate are the most predominant in chicken. Other compounds identified were methyl palmitoleate and methyl stearate. As can been seen from the table, the FAME composition for both the control oil sample and enzymatically hydrolyzed sample prior to extraction were similar. Methyl myristate was detected at low percentage in samples enzymatically pretreated for 2 h and 3 h. Table 3.3 presents the major fatty acids identified in the pork samples (both enzyme pretreated and control sample). Methyl oleate and methyl palmitate are the most predominant methyl ester identified. Others FAME identified include methyl palmitoleate, methyl stearate and methyl linoleate. Metyl myristate was not identified in the control samples but was present in

small amounts 5.96-7.29% in the enzyme pretreated samples. Enzymatic pretreatment had no effect on the FAME composition of chicken and pork fat versus control samples. However there were traces of other FAME derivatives which could not be identified in the enzymatically treated in the chicken samples. The experimental results are in agreement with the literature data for major fatty acids identification for chicken and pork (Hui, 1996)

Conclusion

The effect of enzymatically pretreatment of chicken and pork fat prior to solvent extraction has been demonstrated. Enzymatically hydrolyzed significantly increased the amount of oil yield obtained as compared to control samples. Enzymatic hydrolysis for 2 h gave and increased oil yield of 16.2% and 21.7% in pork and chicken samples respectively. There was a significant difference between enzyme pretreated samples and control samples $P<0.05$. The enzymatic hydrolysis of animal fat prior to extraction can also be applied to other oil extraction method such as rendering to give an increased oil yield. Microwave extraction of this animal tissue also proved to be effective based on the amount of oil yield reported. Enzymatic hydrolysis has an effect on improving the oil yield in the samples thus reducing the extraction period. The results obtained were comparable to that obtained by soxhlet extraction.

Table 3.1: Comparison between microwave assisted extraction and soxhlet extraction methods

	Pork			Chicken		
	1 h (%)	2 h (%)	P value	1 h (%)	2 h (%)	P value
[1]Microwave	85.50± 0.2	93.09±.0.04	(<.0001)	88.36± 0.4	90.44±0.5	(1.000)
[2]Soxhlet	83.82±0.3	84.35±0.7	(1.0000)	88.19±0.6	88.77±0.4	(1.000)
P value	0.0320	0.0017		0.0037	0.0013	

[1] intermittent irradiation (180 s on 90 s off 270s on) total irradiation time = 7.5 min

[2]Soxhlet extraction for 1 h 30 min (45 min immersion, 60min rinsing, 45 min evaporation)

3 numbers of observations

Table 3.2 Hydroperoxide value and *p*-anisidine value of pork and chicken oil samples (both enzyme pretreated and control)

Samples	Hydroperoxide value (mmol CuOOHeq/kg)	*p*-anisidine value (%)
Pork samples		
Control	11.14±1.25	14.38
30 min	7.66±0.66	5.78
1 h	5.69±0.69	4.30
2 h	4.86±0.20	3.28
3 h	4.78±0.02	2.57
Chicken samples		
Control	18.27±3.49	21.35
30 min	16.46±0.14	11.75
1 h	19.87±2.06	23.30
2h	41.76±1.75	59.65
3h	21.93±0.83	56.93

3 numbers of observations

Table 3.3: Fatty acid composition of oils samples enzyme pre-treated prior to solvent extraction.

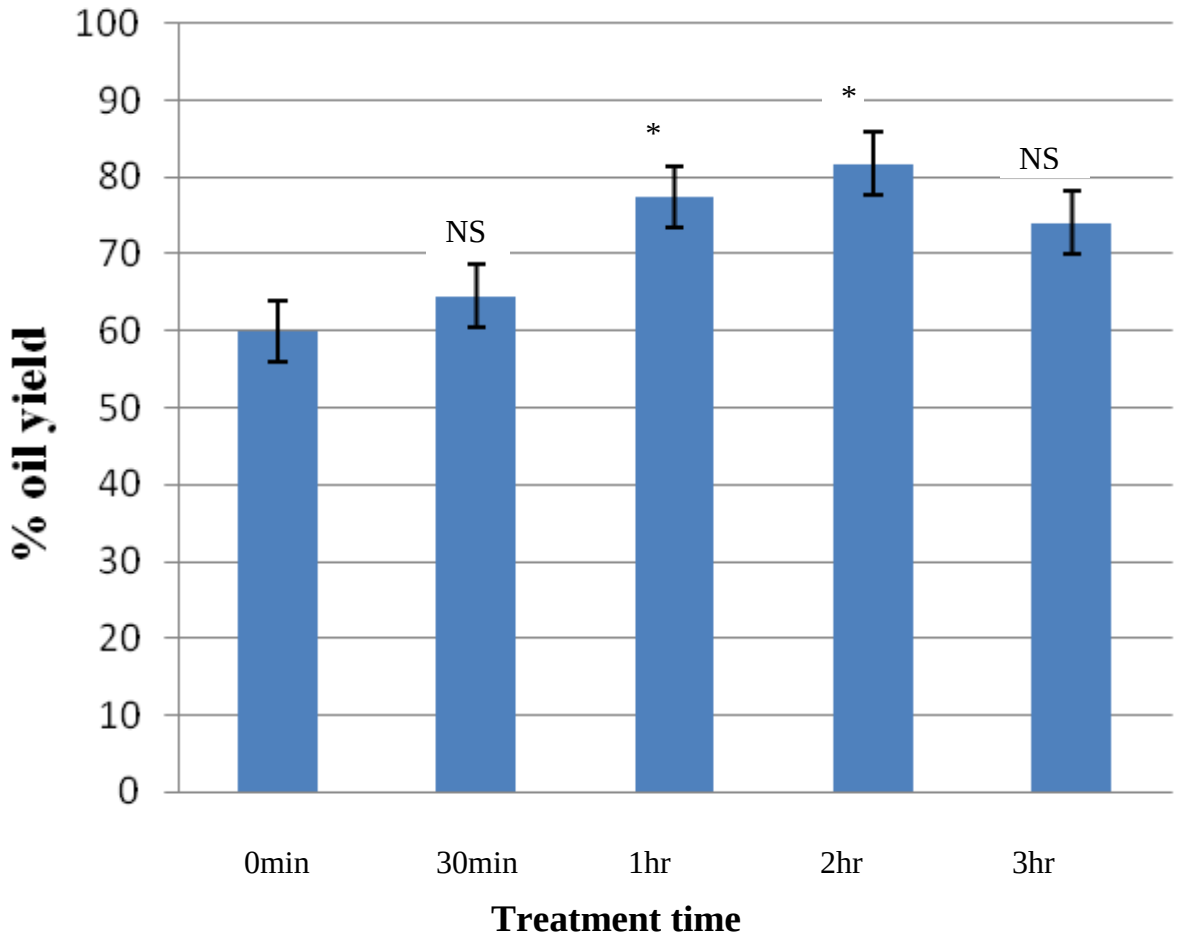
TRT[1]	C14:0	C16:0	C16:1	C18:0	C18:1	C18:2
			CHICKEN			
0 Min (%)	ND*	30.22	4.32	42.19	14.89	14.89
30 min (%)	ND*	27.23	8.76	4.47	41.15	14.23
1 h (%)	ND*	27.02	7.01	6.59	39.74	14.53
2 h (%)	1.09	28.31	6.72	6.40	41.57	15.90
3 h (%)	1.04	27.33	8.03	3.99	40.52	15.04
			PORK			
0 Min (%)		31.38	2.79	12.26	40.03	9.26
30 min (%)	5.96	27.23	3.51	11.77	39.38	9.07
1 h (%)	6.23	25.57	3.71	11.61	39.35	9.79
2 h (%)	7.29	29.37	3.81	12.12	43.60	10.64
3 h (%)	6.47	25.08	3.52	12.37	40.10	8.80

[1] Treatment time

*Not detected

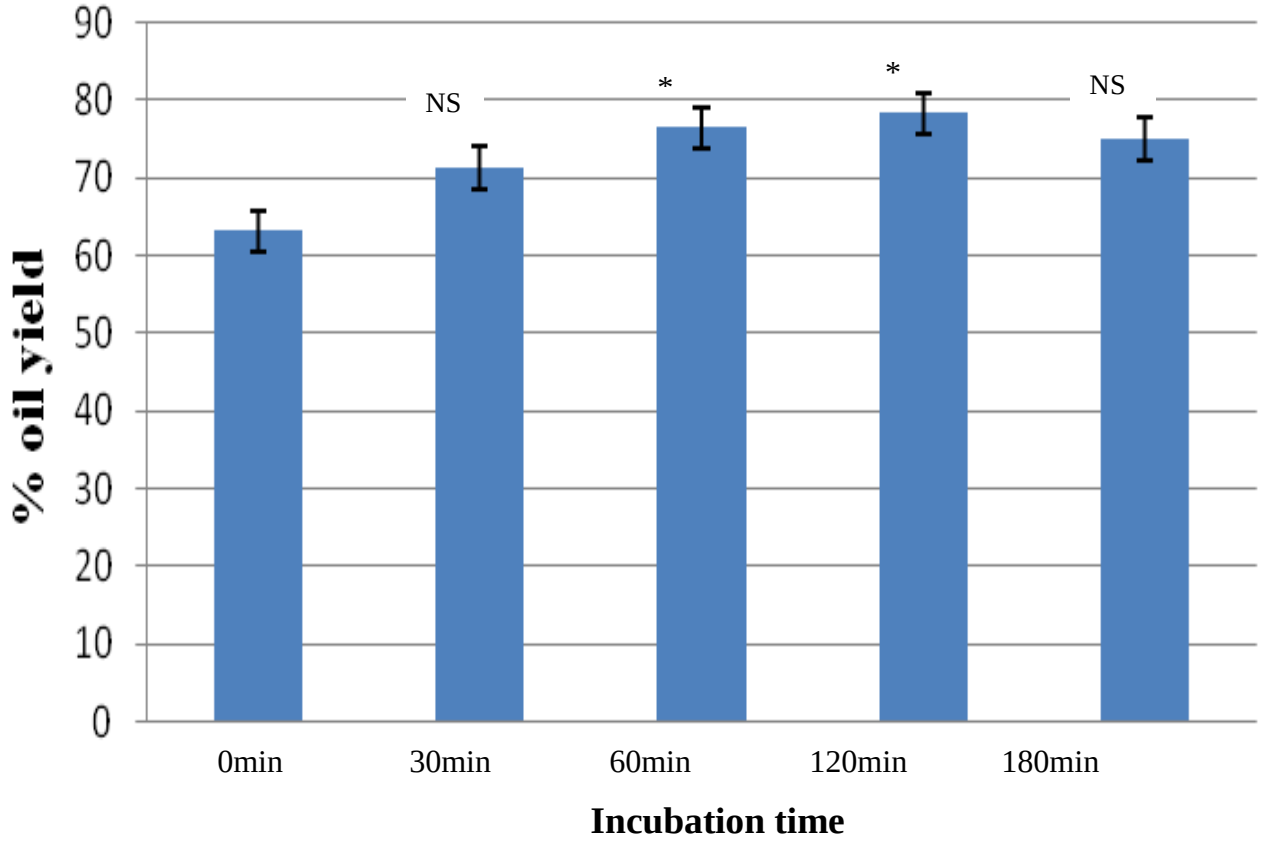
Composition not significantly different P>0.05

Figure 3. 1 Optimization of protease pretreatment for oil yield in chicken samples



NS: no significant difference from 0 (P>0.05) * Significant difference from 0(P<0.05)

Figure 3. 2 Optimization of protease pretreatment for oil yield in pork samples



NS: no significant difference from 0(P>0.05) * Significant difference from 0 P<0.05

Figure 3. 3 Optimization of oil yield from chicken fat discard using microwave assisted extraction

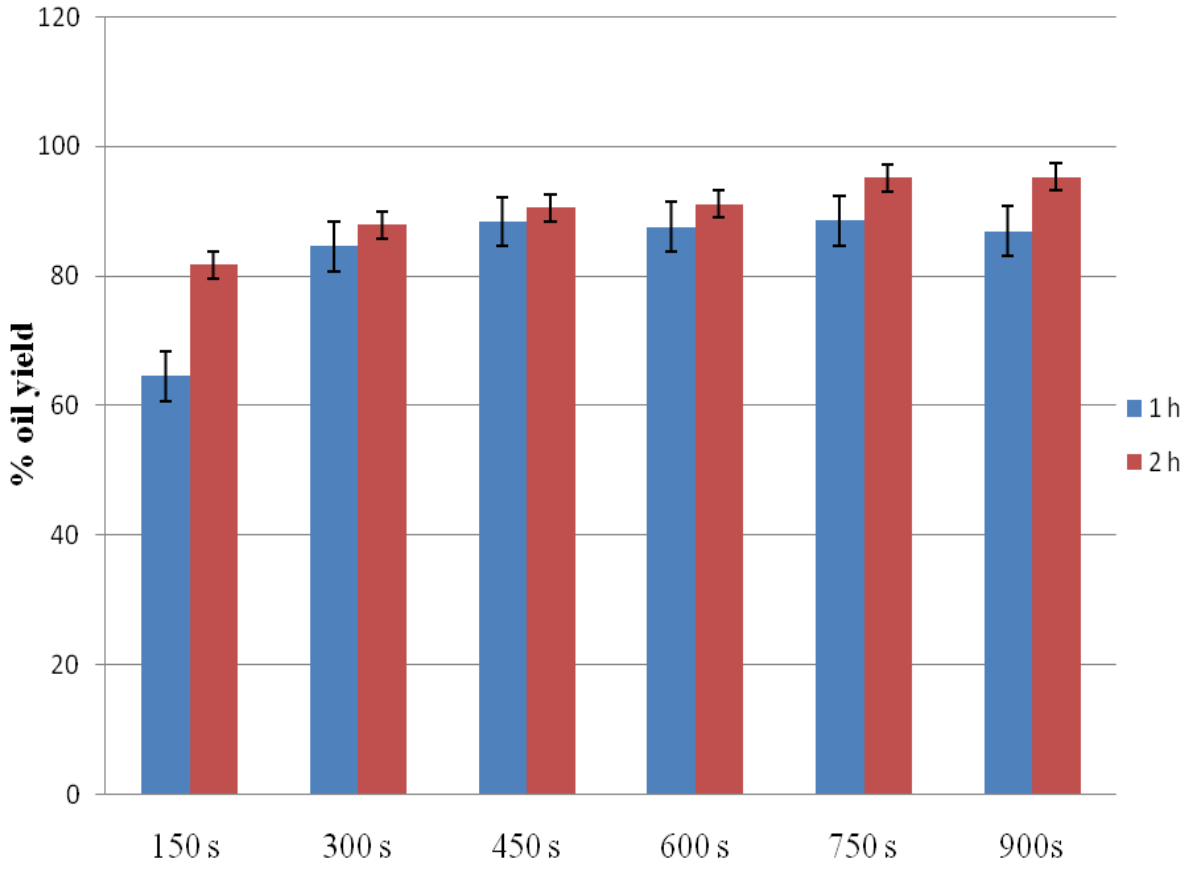
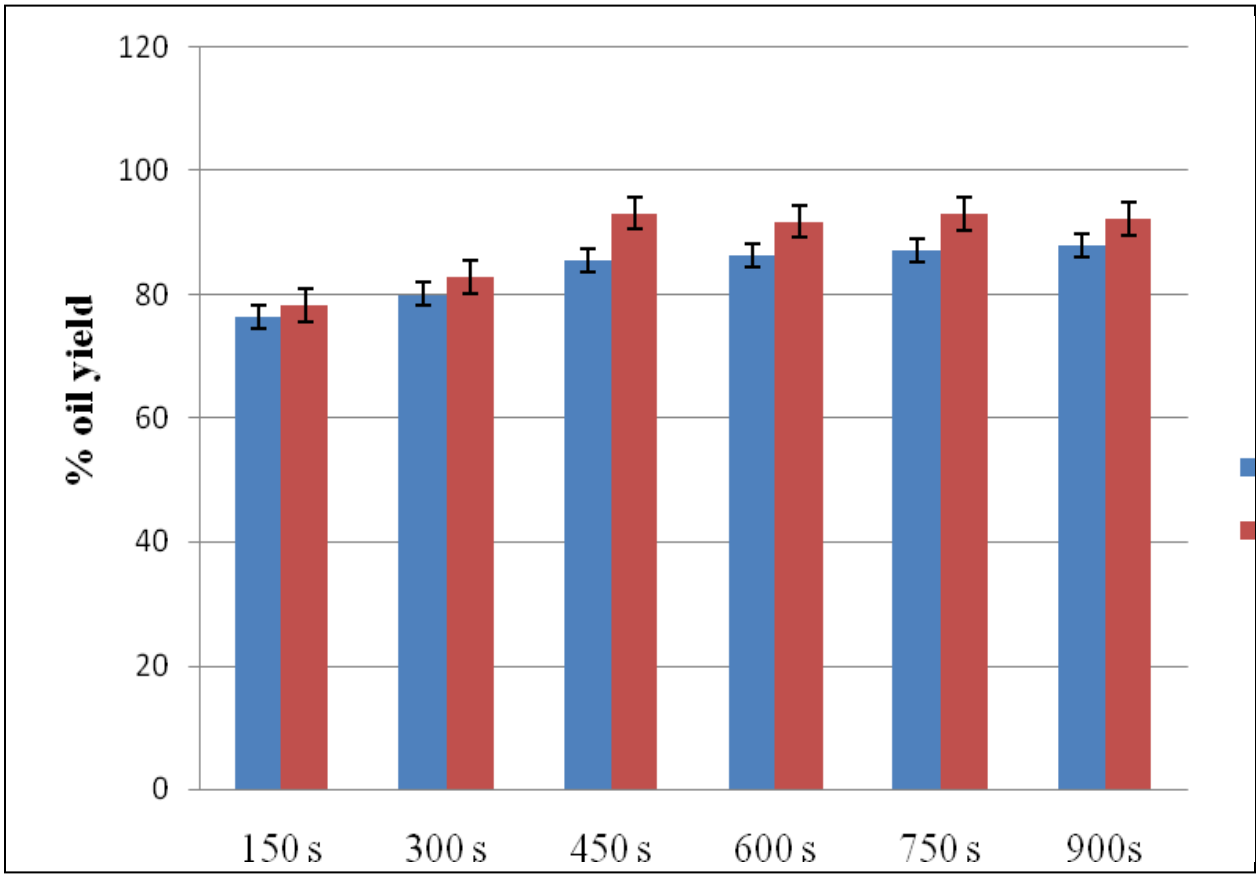


Figure 3. 4 Optimization of oil yield from pork fat discard using microwave assisted extraction



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Connecting statement

The first manuscript investigated the use of enzymatic hydrolysis to increase fat yield from animal processing discards. The results showed that the enzyme pretreament for 2h increased the fat yield of the samples.

The second manuscript looked at the use of pulsed electric field as a pretreament of animal fat discard prior to solvent extraction to increase fat yield and result obtained are compared with oil yield from conventional soxhlet extraction method.

Chapter 4: Pulsed electric field pre-treatment and microwave assisted extraction of oil from animal discards

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ABSTRACT

Pulsed electric field (PEF) was investigated as a pretreatment step of increasing the yield of fat from pork slaughtering discards for subsequent production of biodiesel. The effect of PEF on oil quality was also studied. Pulsed electric field strenght varied from 2 kv cm^{-1} to 6 kv cm^{-1} and was applied at 1Hz for 1 min as a pretreatment prior to microwave assisted extraction (MAE) using hexane. The extracted oils were analysed for hydroperoxide and p-anisidine values as indices of the extent of oxidation in the fat. The fatty acid methyl ester (FAME) profiles of the oil samples were determined using gas chromatography. Fat yield was determined gravimetrically after extraction with either MAE or by the conventional method using soxhlet. Using MAE, PEF strength of 4 kv cm^{-1} enhanced fat yield, with an increase of 7% compared to control (0 kv cm^{-1}). The quality assessment of the oil extracted with PEF pretreated samples showed no effect of PEF on the quality of the oil when compared with control samples. There were no significant differences (P=1.0000) in the hydroperoxide value and p-anisidine values between the pretreatments and the control control. PEF pretreatment had no effect on the fatty acid composition of the fat.

Key words: PEF (pulse electric field) pretreatment, microwave assited extraction, pork

4.1 Introduction

Pulsed electric field is a non-thermal food processing technology. It involves the application of external electric field to induce critical electrical potential across a cell membrane. The field strength applied causes an electrical breakdown and structural changes that increases the permeability of cell membranes. It has been proposed as a pretreatment step for oil seeds, and has been used for the preservation of foods such apple mash (Guderjan et al., 2004).

PEF enhances mass transfer processes within plant or animal cellular tissues, as the permeability of cytoplasmic membranes are altered during treatment (Knorr, *et al., 1998*). Mass transfer rates are enhanced by electroporation of plant cell membranes improving tissue softness and influencing textural properties. Biological samples such as carrots, potatoes and apples treated with PEF lose their water content more rapidly during osmotic drying when they were subjected to PEF (Angersbach, *et al.*,(2002); Lebovka, *et al.,* 2004).PEF has also been used to enhance juice production, increasing the content of valuable components and even replacing the enzymatic maceration step (Eshtiaghi, *et al.,* 2000).

Other methods that have been used as pre-treatments prior to solvent extraction include mechanical pressing and enzymatic hydrolysis to increase the fat yields from seeds and other oil bearing materials. Due to long heating periods**,** the quality of oil is sometimes affected and the use of enzymes as a pre-treatment has not been commonly used due to high cost of these enzymes. The use of pulsed electric field as a pre-treatment of oil seeds prior to mechanical pressing has been investigated. The application of PEF in the range of nanoseconds to microseconds leads to a permeabilisation of biological membranes. Dependent on field strength, pulse duration, and pulse number, the permeabilisation achieved can be reversible or irreversible (Gurdejan et al., 2007).

Zimmermann, *et al.,* (1974), reported a reversible structural changes of cell membranes with short pulse duration of 20 ns–10 ms and a field strength of 1–10 kV/cm. Pores, formed by electroporation, were resealed and the membrane conductivity reverted back to the original state. This effect can be used to induce stress reactions and activate the production of metabolites.

Extending the pulse duration to 10–15 ms at the same field strength or higher number of pulses resulted in an irreversible damage of the membrane, causing a loss of the cell hardiness (Sale *et al.,* 1967). This application is mostly used for microbial inactivation and for increased juice and/or oil yields in biological samples.

Microwave assisted process is a novel method for extracting soluble products into a fluid from different materials using microwave energy. Intact organic and organometallic compounds can be extracted more selectively and more rapidly with similar or better recovery when compared to other methods (Pare, *et al.,* 1997). Microwave assisted extraction consumes low amount of energy, smaller volumes of solvents and uses less toxic solvents hence smaller quantities of waste products are generated . This makes it more advantageous to use over conventional extraction technologies.

The present study was aimed at using PEF as a pretreatment prior to microwave assisted extraction. PEF application is useful and can be applied on an industrial level to improve oil yield, to verify its capacity and to verify the quality of the oils thus recovered.

4.2 Materials and Methods

4.2.1 Sample Preparation

The pork processing discards were obtained from a local supermarket in Quebec. Non fatty tissues such as bone were hand removed. In order to get a homogeneous sample the source material were cut into pieces manually with a knife on a chopping board. All samples were drawn from the same batch.

4.2.2 PEF (Pulsed Electric Field) pre-treatment

Pulsed electric field treatment was applied using a PurePulse (PurePulse Technologies, San Diego, USA) exponential decay pulse generator with a maximum voltage of 10 kv cm^{-1} and a maximum average power of 8 kW. About 4 g of the source material were weighed into the PEF treatment chamber. The peak pulse voltage used was 9 kV, resulting in electric field strength of 3 kV cm−1. A series of 60 pulses were applied at ambient (22^0C) temperature to obtain a specific energy input of 10 kJ kg^{-1}. The pulse repetition rate was 1 pulse sec^{-1} for a total treatment time of 1 min. The samples were transferred into glass tubes and the treatment chamber was rinsed 3 times with hexane to recover residual in the treatment chamber. Oil extraction from the PEF and the control samples was carried out using microwave assisted process.

4.2.3 Microwave assisted extraction

Oil was extracted from the PEF treated and the control samples using microwave assisted process. The original weight of the sample (prior to PEF treatment) was recorded as W_1. The samples were transferred into a 250 ml quartz extraction vessel and fat extraction was performed using a microwave reactor SYNTHEWAVE 402 Prolabo, USA. The vessel was inserted in the extraction cavity, fitted with a condenser and irradiated in the following sequence at full power (300 W): 60 s on, 30s off, and 90 s on for a total irradiation time of 3 min. The temperature of the extractant was measured before and after irradiation. After irradiation, the glass vessel was washed with 10 ml of hexane and the washes were pooled in the extraction vessel. The extract was filtered under vacuum through a whatman filter cup and collected in glass bottles with Teflon screw caps. The weight of the glass bottles was recorded as W_2. The residue in the extraction vessel was washed with 20 ml hexane and filtered and the filtrate was added to the

extract. The combined extracts in the glass bottles were evaporated under nitrogen with heating at 40^{o}C. The samples were kept in vacuum desiccators overnight. After the solvent had evaporated, the bottles containing the oil were reweighed. The % oil yield was calculated as follows:

$$\% \text{ oil yield} = \frac{\text{Weight of Oil}}{\text{Weight of sample}} \times 100 \quad \text{..... (4.1)}$$

Fat determinations were performed in triplicate.

4.2.4 Soxhlet extraction

Soxhlet extraction was carried out using SER 1[illegible] VELP ® Scientifica, UK. About 5-10g of the sample without PEF treatment were weighed into thimbles. The extraction glass vessels were weighed and 60 ml of hexane were added into the extraction glass vessels. The soxhlet extraction program was set at Immersion 1 h, rinsing for 1 h followed by evaporation for 45 min. The extraction was carried out at 180^{o}C. After soxhlet extraction, the glass vessels were placed in a 300^{o}C oven to complete solvent evaporation for 30 min. The extraction glass vessels were then cooled in a desiccator and the % oil yield was calculated as follows using equation 4.1.
Fat determinations were performed in triplicate

4.2.5 Quality analysis

Oil was extracted from the PEF pretreated samples and non PEF treated samples using a soxhlet extractor with slight at the hexane recovery step. The solvent was evaporated under nitrogen after which the oil were placed in an air tight sealed glass bottles, labelled and stored in the freezer at -53^{0}C for further analysis.

4.2.5.1 Hydroperoxide determination

The modified xylenol orange method to evaluate the formation of lipid hydroperoxides proposed by Eymard and Genot (2003) was used. In this procedure ammonium ferrous sulphate 2.5mM was dissolved in 250 mM H_2SO_4. Next, 25 ml of this solution were mixed with 19 mg of xylenol

orange previously dissolved in 50 ml of methanol. The solution was made up to a final volume of 250 ml with methanol Final concentrations of xylenol orange, ammonium ferrous sulphate, H_2SO_4 were 100 µM, 0.25 mM, and 25 mM, respectively. The reagent was prepared just before use.

For the assay, 25 µl of animal oil were pipetted into microtubes (1.5 micro locking tubes) and made up to 150 µl with methanol. Then 1350 µl of FOX2 reagent were added to the oil samples in the micro locking tubes. Final concentrations of ferrous ammonium sulphate, H_2SO_4, and xylenol orange in the reaction mixture were 0.22 mM, 22.5 mM and 90 µM, respectively in water/methanol (10/90;v/v). The reaction mixtures were thoroughly mixed and then incubated in darkness at 22^0C for 50 min. Their absorbencies were read at 560 nm against a blank (150 µl methanol and 1,350 µl FOX2 reagent).

A standard curve was developed with solutions of cumene hydroperoxide (CuOOH) in methanol. Concentrations in reaction mixture were 0, 10 and 15 and 20 µM. The standard curve was used for the conversion of absorbance at 560 nm into concentration of CuOOH equivalents (µM) in the reaction mixture. The levels of hydroperoxide were expressed as milli mol (mM) of cumene hydroperoxide equivalents/kg of oil.

4.2.6 Measurement of P-Anisidine Value

The *p* –anisidine value was measured using the AOCS official method CD 19-80 as follows:
The oil sample was weighed (0.5 - 4.0 g) into a 25 ml volumetric flask and dissolved and diluted to volume with isooctane. The absorbance of the solution was measured at 350 nm with isooctane as blank. Next, 5 ml of the fat solution were pipetted into a 10 ml Teflon lined screw capped test tubes while exactly 5 ml of the solvent (isooctane) were pipetted into a second test tube as a blank. Then 1 ml of the p-anisidine solution was added to each test tube and shaken. After exactly 10 min the absorbance of the solution in the first test tube was measured using the solution in the second test tube as blank.

4.2.7 Fatty Acid Methyl Ester profiles (FAME profile)

The FAME were prepared using sodium methylate as catalyst according to Fournier *et al.,* (*2006*). About 20 mg of the oil sample were weighed into a 30 ml Teflon lined screw capped test tube. Next, 1 ml of toluene and 2 ml of a 0.5 M sodium methylate solution in methanol were added. The solution in the test tube was then incubated in a water bath at 50°C for exactly 5 min. After cooling, 100 µL of glacial acetic acid was added using a glass syringe, followed by 5 ml of hexane and 5 ml of distilled water. The test tube with its content at 4^0C was centrifuged for 3 min at 2000 rpm. The hexanic phase containing fatty acid methyl esters was pipetted into glass bottles with Teflon lined screw caps. The aqueous phase is extracted a second time with 5 ml of hexane at 4^0C then centrifuged for 3 min at 2000 rpm. The two hexanic layers were pooled together then dried under a flow of nitrogen. The fatty acid methyl esters were then diluted to a concentration of 0.1 mg/ml with hexane.

4.2.8 Gas-Liquid Chromatography analysis

The FAME were analysed using a Hewlett-Packard Model 5890 capillary gas chromatograph (Palo Alto, CA, USA) with a DB-23 60m 0.25 ID 0.15 micrometer film thicknesses,using a flame ionization detector (FID).

4.2.9 Statistical analysis

The data obtained from the studies were analyzed using SAS version 9.13 for windows (SAS Institute, Cary, NC, USA). The data were tested for normality and were considered normal if it had a P value greater than 0.05 according to the Shapiro-Wilk statistics. Analysis of variance (ANOVA, SAS PROC GLM) was used to analyze the normally distributed data. All results were reported at a 0.05 significance level.

4.3 Results and discussion

4.3.1 Effect of pulsed electric field (PEF) pretreatment on oil yield

Figure 4.1 presents the result of oil yield obtained from sample pre treated with PEF prior to microwave assisted extraction. Various field strengths were used while keeping the pulses constant. Sixty (60) pulses were used because it was observed that pulses greater than 60 caused high disintegration of the cell structures; this could be a potential fire harzard because there is oil leakage from the chamber. . From figure 4.1 it can be seen that the effect of PEF pre treatment is greater at 4 kv cm^{-1} with an oil yield of 58.98% versus an oil yield of 52.13% obtained with the control samples. PEF pretreatment at field strength of 4 kv cm^{-1} resulted an increase of 6.85% and this increase was significantly different ($P = 0.0344$) compared to the control. A decrease in oil yield was observed with samples pretreated at electric strength of 6 kv cm^{-1}. This decrease could be due to the high impact of the field strength causing high disintegration in the cell structure of the sample, thus extracting the oil which might be lost in the chamber or leaked out, prior to microwave assisted extraction and thus a reduced oil recovery. There was a significant difference between oil yield at pretreatment electric field of 4 kv cm^{-1} and 6 kv cm^{-1} ($P<0.0001$). There were no significant difference between oil yield with the 2 kv cm^{-1} pretreatment versus control samples ($P=1.000$). PEF pretreatment was effective in improving the oil yield using microwave assisted extraction. Oil yield using MAE was highest at 4 kv cm^{-1} PEF pretreatment. Irreversible permeabilisation at electrical field strengths higher than 1 kV cm^{-1} produces permanent pores, which result in improved mass transfer and enhanced extraction of oil. The main objectives of a successful oil recovery are the rupture of cell walls, to obtain diffusion of oil components as well as to obtain final separation of oil (Carr, 1995). As the data show, PEF could be used as a pre-treatment for cell disruption prior to oil extraction.

4.3.2 Microwave Vs Soxhlet

Figure 4.3 presents the results of oil yield obtained with microwave assisted extraction versus soxhlet extraction. The samples used were not subjected to PEF pre treatment. The microwave assisted process was applied for 3 min with initial temperature of 41^{o}C and final temperature of

58^{o}C. The soxhlet extraction was carried out for 3 h at 180^{o}C. Microwave assisted extraction for only 3 min gave an oil yield of 52.13% versus a yield of 64.2% obtained with the soxhlet extraction for 3 h (P=0.005). The increase in oil yield obtained using soxhlet was 12% with the considerable time difference of 2 h 57 min. The effect of microwave in enhancing oil extraction from the samples due to the presence of water (high dielectric property) permits a high ability to absorb microwave energy as opposed to bound water molecules which are characterized by a lower dielectric constant (Ganzler *et al., 1997).* Localised heating leads to the expansion and rupture of cell walls and is followed by the liberation of essential oils into the solvent. However when PEF pretreatment at an electric field strength of 4 kv cm^{-1} was applied there were no significant difference (P=0.1411) in fat yield when compared with soxhlet extraction for 1.5h.

4.3.3 Quality analysis

Hydroperoxide and p-anisidine values of the oils recovered from pork discrds were used to measure the extent of oxidation. Oxidation in lipids starts with the production of unstable intermediary compounds such as free readicals and hydroperoxides. These compounds further decompose into secondary compounds such as aldehydes, ketone, and alcohols, which are resoponsible for the production of off flavours (Ladikos *et al,* 1990). To evaluate oil quality both primary and secondary products resulting from the lipid oxidation were quantified. Table 4.1 presents the results of the oxidation assessment. It can be seen that the hydroperoxide value in the PEF pretreated samples decreases with increased electric field strength. Hydroperoxide values of 2 kv cm^{-1}, 4 kv cm^{-1}, 6 kv cm^{-1} were 6.66, 5.56 and 4.96 respectively. There were no significant difference in hydroperoxide value within the treatments (P=0.1237). Hydroperoxide value for the control samples was 5.04. This could be an indication of oxidation prior to pretreatment. Guderjan *et al*, (2004) indicated that, it is possible that pulsed electric fields have an oxidative effect on oil quality. Long storage time and temperature are among factor causing oxidation of lipids. The p-anisidine value indicates the production of secondary oxidation products (Akoh and Min, 2002) in the samples. P-anisidine value at 2 kv cm^{-1} electric field strength was 11.45% increasing to 14.33% at field strength of 6 kv cm^{-1}, indicating that secondary oxidation products were present in samples at electric field strength of 6 kv cm-1. This

could be due to the effect of temperature due to the caused by the high field strength. There are no significant difference in p-anisidine value of the oil sample (P=0.6100)

4.3.4 FAME composition

It is known, that different extraction methods of oil affect the composition and concentration of minor components of oil. Table 4.2 compares the fatty acid composition of oil samples extracted from PEF pretreated samples versus the nontreated. As shown in the table, there were no statistically significant differences in the FAME profiles of PEF pretreated samples versus the control samples. The table further shows that C16:0, C18:1 and C18:0 were the predominant fatty acids. Other fatty acids identified include C14:0, C16:1, C18:2. This FAME profiles are consistent with FAME profiles of pork reported in the literature (Hui, 1996).

Conclusion

In this study the effect of PEF was investigated as a means of pretreatment of pork processing discards prior to MAE of the fat. Maximum fat extraction yield was achieved at PEF strength of 4 kv cm^{-1} and 1 Hz for 1 min. The PEF pretreatment had a significant difference (P=0.0344) in oil yield when compared with non PEF pretreated samples. The results from this study shows that oil yield using MAE can be improved by the application of PEF. PEF pretreatment had no effect on the FAME composition. The results also show that pulsed electric fields could be used to increase oil yield from pork processing discards with no adverse effect on the quality of the oil as measured by hydroperoxide value and p-anisidine values.

Table 4.1: Hydroperoxide value and *p*-anisidine value of Pork oil pretreated with PEF prior to solvent extraction

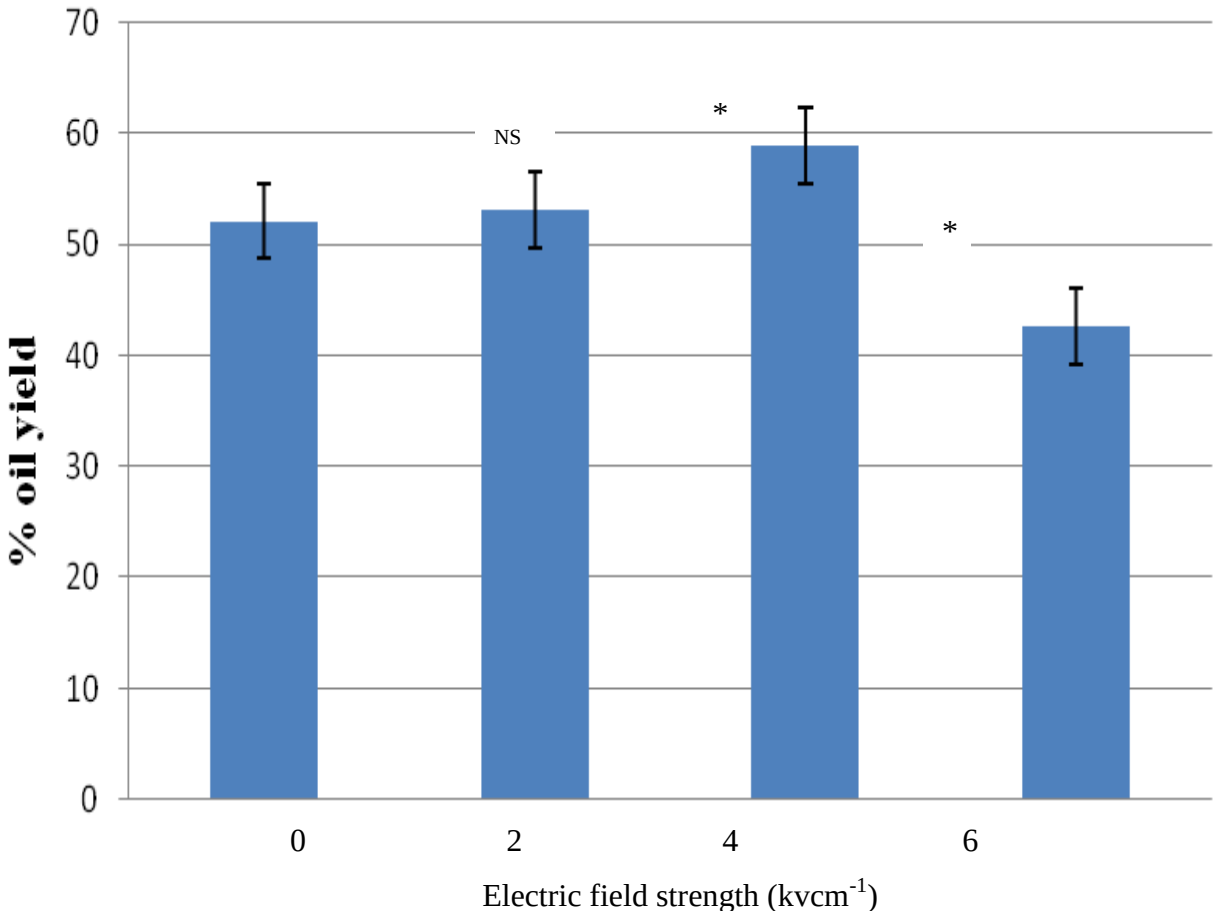
Electric field strength ($kV\ cm^{-1}$)	*p*-anisidine value (%)	Hydroperoxidevalue (mmol CuOOH eq/kgoil)
2	17.61±1.15	6.66±1.87
4	11.45±1.98	5.56±0.23
6	14.33±0.82	4.96±0.17
0	17.61±1.34	5.04±0.12

3 numbers of observations

Table 2: Fatty acid composition (%) of Pork fat (W/ %) PEF pretreated prior to solvent extraction

	PEF strength ($kvcm^{-1}$)			
Fatty acids	0	2	4	6
C14:0	6.7	6.4	6.9	5.9
C16:0	25.9	24.9	26.3	24.8
C16:1	2.7	3.4	3.41	3.3
C18:0	12.4	11.9	12.30	11.8
C18:1	38.9	36.8	38.3	38.4
C18:2	9.6	8.9	9.5	9.43

Figure 4.1 Effect of PEF pretreatment on oil yield



NS: no significant difference from 0 $Kvcm^{-1}$ ($P>0.05$)

*significantly different from 0 $Kvcm^{-1}$ ($P<0.05$)

Figure 4.3: Soxhlet extraction versus Microwave assisted extraction

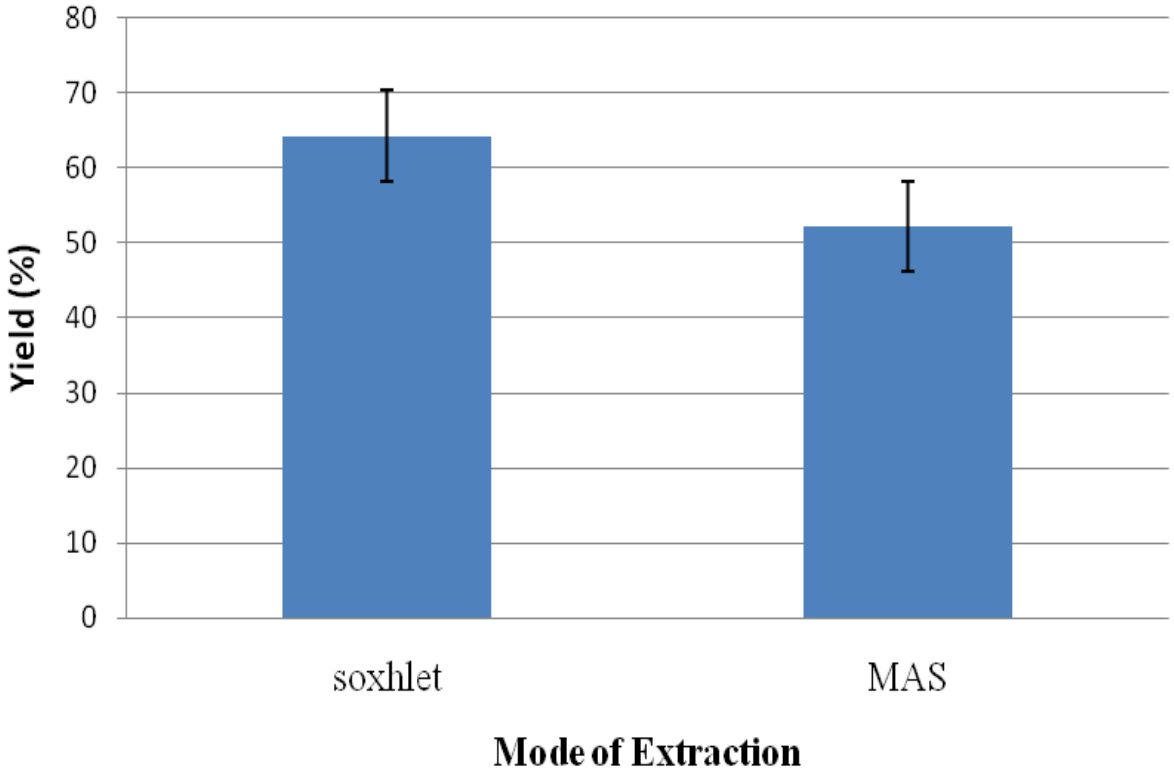
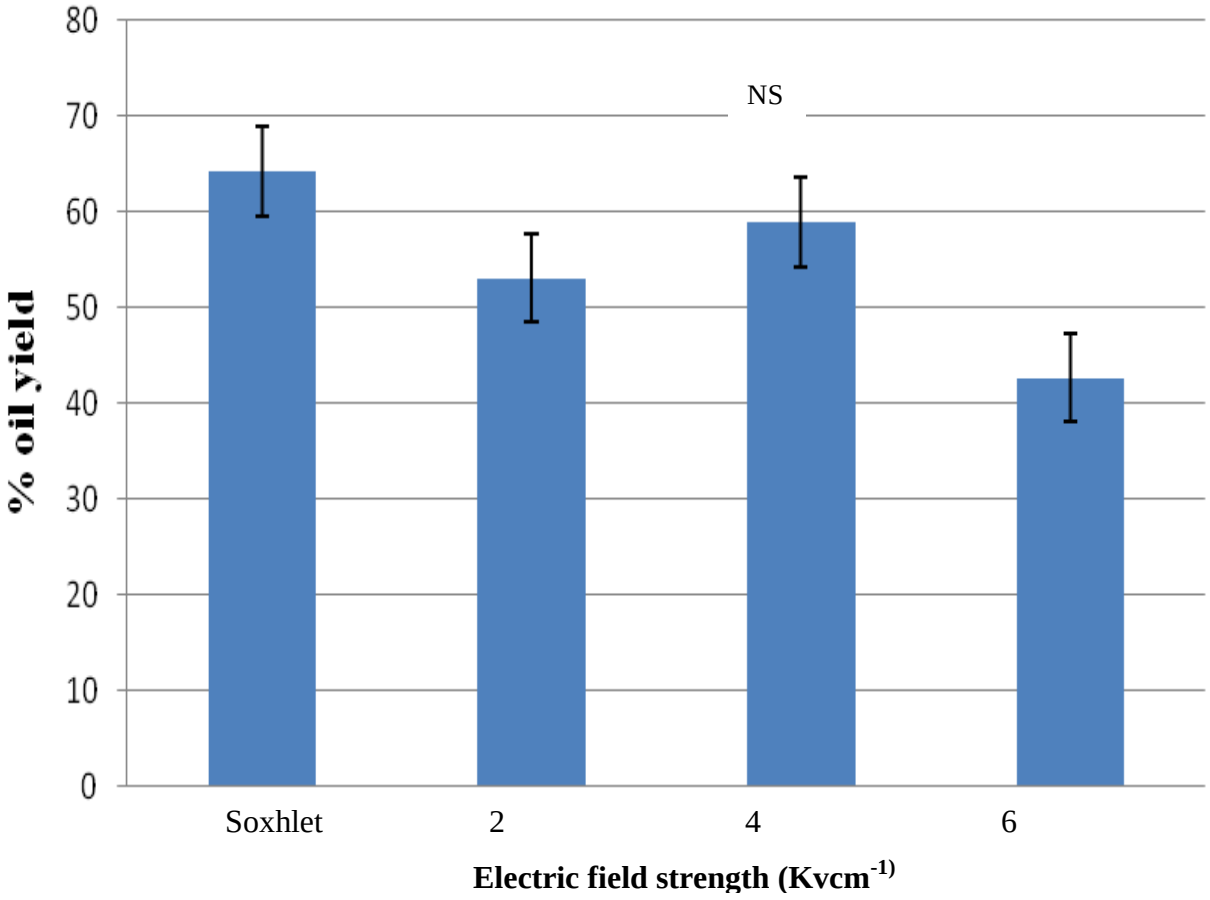


Figure 4.2: Effect of PEF pretreatment on fat yield using MAE versus soxhlet extraction



NS: no significant difference with soxhlet (P>0.05)

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CHAPTER 5: GENERAL CONCLUSION

Oil was extracted from chicken and pork fat discard. Enzymatic pretreatment and pulsed electric field pretreatment was used prior to solvent extraction by microwave assisted extraction using hexane. When compared with control samples, enzymatically pretreated samples produced a maximum oil yield of 20% in chicken samples and an increase in yield of 16.6% in pork samples. An increased in oil yield was observed with the different time intervals (30 min, 1 h, 2 h), however there was a decrease in oil yield at 3 h. Enzyme pretreatment of animal discard prior to solvent extract proved to be effective in increasing oil yield during solvent extraction. The effectiveness of microwave assisted extraction was also demonstrated in this study. Optimization of microwave assisted extraction process produced a maximum oil yield of 81.7 and 71.23 in chicken and pork samples respectively from samples pretreated with enzymes. The effect of pulsed electric field pretreatment was also seen in the study. The study shows that pulsed electric field pretreatment is effective in improving the oil extracting using microwave assisted extraction. Microwave assisted extraction for 3 min produced oil yield comparable to soxhlet extraction for 3 hr. Advantages of microwave assisted extraction includes shorter period of extraction and reduced solvent consumption. Other researchers have studied the effect of PEF pretreatment on oil extraction from oil seeds as well as enzymatic pretreamt. The result obtained from this study also proved that both enzyme and PEF pretreamt are effective methods that can be used to optimize extraction of oils from animal tissue. The result from the study shows that enzyme pretreatment was more effective in in increasing the oil yield using MAS as compared to pulsed electric field pretreatment. However oil extraction of pulsed electric field pretreatment samples using MAS was not optimized. Future work should look into optimizing the effect of PEF pretreatment and also optimize MAS condition for best oil yield.

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