Plasticization of kafirin films

by

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SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

MSc (Agric) Food Science and Technology

IN THE
DEPARTMENT OF FOOD SCIENCE
FACULTY OF NATURAL AND AGRICULTURAL SCIENCES
UNIVERSITY OF PRETORIA
SOUTH AFRICA

OCTOBER 2004

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DECLARATION

I declare that this dissertation is my own work. It is being submitted for the degree M.Sc (Agric) Food Science and Technology at the University of Pretoria. It has never before been submitted for any degree or examination at any other University or Technikon.

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ABSTRACT

Despite the potential of kafirin (sorghum prolamin protein) films, up until now there has been no in-depth investigation on the plasticization of kafirin films, similar to that done on zein films. Since protein films tend to be very brittle, plasticization is a very important aspect.

Cast films were produced from kafirin and plasticized with different combinations of plasticizers (glycerol (G), polyethylene glycol 400 (PEG) and lactic acid (LA)) according to a rotatable central composite statistical design. The effects of the different plasticizer combinations on the film properties (tensile-, T_g-, moisture and oxygen barrier properties), were investigated through a series of tests performed on the films. Plasticization of kafirin films was investigated further by determining the effect of an emulsifier, diacetyl tartaric ester of monoglyceride (DATEM) and an acidulant, glucono-δ-lactone (GDL) on the films. To investigate the distribution and migration of the plasticizers in kafirin films, the films were studied by light microscopy.

It was clear that G, PEG and LA together were necessary to plasticize kafirin films. G and PEG were found to be effective plasticizers, leading to a decrease in film strength and an increase in strain as the plasticizer amount increased. LA was, however, found to act rather as a solvent for kafirin during film casting, instead of acting as a plasticizer. An increase in plasticizer content also brought about a lowering in the T_g of the films, as well as an increase in film permeability to water vapour and oxygen. G and PEG were found to attract water from the atmosphere, which proved to be very influential on the properties of the films; the more plasticizer present in the film, the more moisture attracted, the greater the effect on the film properties. DATEM was not found to be a plasticizer for kafirin films. However, GDL did bring about changes in film properties, similar to G and PEG. It caused film strength to decrease and film strain to increase, but with less detrimental effects on the moisture barrier properties of kafirin films. Microscopy showed that the plasticizer migrated over time, apparently leading to plasticizer molecule coalescence, and the formation of plasticizer pools.

The plasticizer combination of G, PEG and LA improved the qualities of kafirin films, reducing film brittleness, but it is not an ideal plasticizer combination due to the fact that it attracts water to the film and it is not stable over time. Moisture is another plasticizer to be taken into account, since it will be absorbed by the plasticizers in high relative humidity areas and will have an additional plasticizing effect on films properties. GDL proved to have potential as a kafirin film plasticizer as it affected the barrier properties of the films less. Further research is recommended into the plasticization mechanism of GDL.

Key Words:
Sorghum, kafirin, films, plasticizer

DEDICATION

Prof. J.D.N. Lotz (1916 –1978), professor in African languages, University of Zululand – the grandfather I have never known but whose legacy has been my inspiration.

ACKNOWLEDGEMENTS

First of all, I would like to express my heartfelt gratitude to the two people that contributed the most to this project and who I will never forget. Prof. John Taylor, my supervisor, for his wisdom, guidance, motivation and patience from the start. Janet Taylor for her help and her friendship.

I would like to thank everybody in the Department of Food Science, everybody that had an ear to listen and a word of advice - it has been a privilege to be part of such a world-class facility. Thank you to Mr. Naushad Emmambux for his help with the films, for sharing his first-hand experience with me! Thank you also to Dr. Riëtte de Kock for showing me the principles behind principal component analysis.

A word of gratitude to the Department of Microscopy, University of Pretoria for the use of their facilities and their assistance. A great thank you to Mr. Alan Hall for his help and patience through all the repetitions of the microscopy sessions with the films. Thank you to the Department of Statistics, University of Pretoria, for all the data they have analyzed, it is greatly appreciated!

Thank you to the European Commission for funding the INCO-DEV contract ICA4-CT-2001-10062, Enviroment-friendly packaging solutions for enhanced storage and quality of southern Africa's fruit and nut export. Thank you to Dr. Mats Stading and everybody at SIK for the analyses done on the films.

Thank you to my family and friends for their support during this time, for being there for me when times were tough.

Thank you to my Father in Heaven for giving me the ability to have made a dream come true!

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1. INTRODUCTION

1.1 Problem statement

Plastics are part of our everyday life, whether you are carrying your groceries home in a polythene bag or sitting on a plastic chair on your porch. Plastics can be made from various carbon sources, e.g. oil, natural gas and coal (Anonymous, 2004). There are about 50 different types of plastics, each with its own specific properties, it just depends on how the specific raw materials are processed. The most frequently used plastics are polyethylene, polypropylene, polystyrene, polyvinyl chloride and polyethylene terephthalate, and none of them are biodegradable (Pavlath & Robertson, 1999). Generally, these materials are disposed of by landfilling. Therefore, other disposal methods, like recycling or methods to just simply use less packaging material, are gaining ground (Potter & Hotchkiss, 1995).

Recently, the South African government imposed a new law on the use of plastic bags (Molamu, 2004). Formerly known as South Africa's "national flower" due to the extensive amount of pollution, the use of plastic bags can now be controlled through this regulation. The main objective was to reduce plastic pollution through the promotion of recycling without costing thousands of people their jobs when the consumption of plastic bags declined. This was put into action mainly by making thicker plastic bags and by making consumers pay for them. This caused consumers to treat plastic bags with greater respect, knowing what they cost. The purchasing of plastic bags also encourages its re-use, which has a positive impact on the environment (Molamu, 2004).

Despite the tremendous efforts made to reduce packaging pollution, it still remains a problem, especially packaging material from the food industry. According to Potter & Hotchkiss (1995), general packaging in the USA constitutes to about 33% of the disposable solid waste, half of which is food packaging. Therefore, it is of cardinal importance that biodegradable packaging materials are developed for the food industry and are put to use. It is not possible for biodegradable packaging materials to replace existing

synthetic packaging materials, but they can act as adjuncts, contributing to the overall quality of products (Kester and Fennema, 1986). This might help to decrease the use of other non-biodegradable packaging materials.

Films or coatings manufactured from natural biodegradable and edible materials, such as proteins, polysaccharides and lipids, are not a new concept and through the years, much research has been done in the field (as reviewed by Kester & Fennema, 1986). What is novel, however, is the possibility of producing films or coatings from sorghum proteins. This could be a means of enhancing sorghum utilization in regions where it is cultivated, especially if the sorghum protein (kafirin) used for film production is extracted from the by-products of the sorghum processing industry, e.g. sorghum bran (Da Silva, 2003). Kafirin, the aqueous alcohol-soluble prolamin protein of sorghum, constitutes about 50% of the total sorghum grain protein (Taylor, Schűssler & Van der Walt, 1984). An application for edible films made from kafirin could be to extend the shelf life and improve the quality of Southern African export fruits and peanuts (Enviropak, 2004).

In general, protein films are very brittle due to the extensive interactions that exist between the protein chains (Krochta, 2002). This can be overcome through the addition of a plasticizer or a combination of plasticizers during film production. A plasticizer is a substance with a low volatility, which will change certain properties of the material it is added to by reducing the intermolecular forces among the polymer chains (Banker, 1966).

The addition of a plasticizer will bring about certain changes in the physical and mechanical properties of the films. The plasticizer will reduce the brittleness of the film, causing a decrease in film strength and an increase in film extensibility (Banker, 1966). In other words, it will make the film more flexible and easier to handle. Plasticizer addition will also affect the barrier properties of the films, normally making the films more permeable to water and oxygen as the plasticizer concentration increases (Park, Bunn, Weller, Vergano & Testin, 1994). Depending on what the film's purpose is, this might be a detrimental side effect. The value of edible films lies in their ability to act

as adjuncts for improving the overall quality of products and by extending product shelf life (Kester and Fennema, 1986). Therefore, if applied to a product, the efficiency of the film will depend on the primary process by which the product it is applied to deteriorates. Whether the reduction in the ability to keep moisture in or oxygen out is detrimental or not, will depend on whether or not it is necessary to ensure the quality of that specific product.

Various aspects of kafirin films have been studied (Buffo, Weller & Gennadios, 1997; Da Silva, 2003; Emmambux, 2004; Taylor, 2003). However, no systematic study has yet been undertaken to investigate the plasticization of kafirin films. The plasticization of cast zein films has been studied in detail (Lawton, 2004). Therefore, this research is an attempt to fill a gap in the research on kafirin films.

1.2 Hypotheses

If kafirin is used to make protein films, the plasticizers found to be suitable for plasticizing zein films, in particular glycerol and polyethylene glycol/polypropylene glycol, will also be effective for the plasticization of kafirin films, because zein and kafirin are similar prolamin proteins (Shull, Watterson & Kirleis, 1991).

Lactic acid will prove to be a useful plasticizer for kafirin films, since it was found to be a primary solvent for kafirin (Taylor, 2003) and a good solvent is a good plasticizer (Fennema, 1996).

Alternative plasticizers, i.e. emulsifiers and/or acidulants will prove to be successful plasticizers, due to kafirin's hydrophobic character (Wall & Paulis, 1978).

1.3 Objectives

- To determine the effect of different plasticizers in combination on the functional properties of kafirin films.

- To determine the effect of the addition of emulsifiers and acidulants as plasticizers on the functional properties of kafirin films.

- To evaluate the plasticizer distribution and migration in the kafirin films.

2. LITERATURE REVIEW

This literature review will give a broad overview on the cereal proteins used for edible film production, with emphasis on the properties and extraction of zein, the prolamin protein of maize and of kafirin, the prolamin protein of sorghum. It will also include the theory of film formation and information on film characteristics. The focus will, however, be on plasticizers and plasticization of edible films – including the theories on how plasticization takes place and different potential plasticizers form protein edible films.

2.1 Cereal proteins: a brief overview

Being at the base of the food pyramid, cereals play an integral part in human and animal nutrition, not only providing carbohydrates but also proteins and other nutrients. When they are compared to legume seeds, cereal grains are relatively low in protein (on average about 10–12% dry basis). Despite this, cereals provide proteins for human and animal nutrition, amounting to almost three times the protein derived from the more protein-rich legume seeds (Shewry & Halford, 2001).

Cereal proteins are not only important from a nutritional point of view, the type and amount of protein present in cereals also influences their functional applications. A very good example is the gluten protein of bread wheat flour (Hoseney, 1994).

According to the original classification of cereal proteins by Osborne (1924), they can be classified into 4 basic types based on their solubility. They are the albumins, globulins, prolamins and glutelins. The albumins are soluble in water. The globulins are soluble in dilute salt solutions but insoluble in pure water and solutions with high salt concentrations. Prolamins are soluble in 70-90 % alcohol and glutelins are soluble in weak acids or bases.

Although the Osborne classification is still in use, cereal grain proteins can also be divided into two classes on the basis of their biological functions

(Lásztity, 1984). Cereal proteins can either be cytoplasmic or metabolically active proteins, or they can be storage proteins. Cytoplasmic and storage proteins differ considerably from each other, both in physical properties and amino acid composition.

Most of the albumins and globulins can be classified as cytoplasmic or metabolically active proteins. They have a relatively small molecular weight and the molecules have a globular form (Lásztity, 1984). The albumins and globulins can be found in high concentrations in the aleurone cells, the bran and the germ of cereal grains. They are also present in the endosperm but in very low concentrations (Hoseney, 1994). The albumins and globulins are high in lysine, tryptophan and methionine, making them nutritionally favourable since these three essential amino acids are generally found only in small quantities in cereals.

The prolamins and glutelins are classified as storage proteins (Hoseney, 1994). They are found mainly in the endosperm of cereal grains because they are stored by the plant to be used during germination by the seedling. The storage proteins, prolamins and glutelins contain high amounts of glutamine and proline, but are low in lysine, arginine, threonine and tryptophan, leading to them having low nutritional value (Lásztity, 1984).

This review focuses on the formation of films made from cereal proteins, with specific reference to zein, the prolamin of maize; and kafirin, the sorghum prolamin. The reason for this is because zein and kafirin are very hydrophobic proteins (Wall & Paulis, 1978) and are potentially useful for packaging materials with a moisture barrier function.

2.1.1 Maize and sorghum prolamin proteins

Esen (1987) proposed a nomenclature for the three major fractions of zein. They are α-zein, β-zein and γ-zein, each of which has its own unique polypeptide composition and solubility properties.

Depending on the maize genotype, α-zein will contribute 75-85% of the total zein and is soluble in 50-95% (v/v) propan-2-ol (Esen, 1987). It consists of M_r 21-25 x 10^3 polypeptides as well as a M_r 10 x 10^3 polypeptide. Beta-zein has two M_r 17-18 x 10^3 methionine-rich polypeptides. It is soluble in 30-85% (v/v) propan-2-ol together with an added reducing agent but it is insoluble in 90% propan-2-ol, making it possible to separate from α-zein. Of the total zein, β-zein constitutes about 10-15%. Gamma-zein contributes about 5-10% of the total zein upon extraction. It is soluble in 0-80% propan-2-ol with a reducing agent and it consists of a M_r 27x10^3 proline-rich polypeptide. Gamma-zein is also soluble in 30% propan-2-ol/30 mM Na ethanoate, pH 6.0 and due to this, it can be separated from α- and β-zein, since neither of them is soluble in 30% propan-2-ol/30 mM Na ethanoate, pH 6.0.

Even though each of the three zein fractions has its own unique amino acid composition, which distinguishes them from one another, it can be said in general that they are low in lysine and tryptophan but rich in proline, glutamine and hydrophobic amino acids (Esen, 1987).

Kafirin constitutes at least 50% of the total sorghum grain protein (Taylor *et al.*, 1984). There are so many similarities in the molecular weight, solubility and structure of kafirin and zein, that a nomenclature for sorghum kafirins was proposed by Shull *et al.* (1991) based on the existing nomenclature of zein (Esen, 1987). The polypeptides with M_r 23 x 10^3 and M_r 25 x 10^3 are the α-kafirins and they are soluble in 40-90% *tert*-butanol together with 2-mercaptoethanol (2-ME) as a reducing agent. The β-kafirins are M_r 20 x 10^3 polypetides and they are soluble in 10-60% *tert*-butanol plus 2-ME. The M_r 18 x 10^3 and M_r 16 x 10^3 electrophoretic bands are also referred to as β-kafirins since they have the same solubility as β-zein. The M_r 28 x 10^3 polypeptides are classified as γ-kafirins. They are soluble in 10-80% *tert*-butanol together with 2-ME. Of the total kafirin fraction, α-kafirin contributes about 80%, the β-kafirin about 7-8% and the γ-kafirin about 9-12% (Shewry, 2002).

Table 1: The characteristics of the different solubility fractions of zein and kafirin

Prolamin	Fraction	Subunit M_r	% of total fraction	Solubility
Zein	α-zein	21-25 x 10^3 10 x 10^3	75-85	50-95% propan-2-ol
	β-zein	17-18 x 10^3	10-15	30-85% 2-propan-2-ol & reducing agent
	γ-zein	27 x 10^3	5-10	0-80% 2-propan-2-ol & reducing agent
Kafirin	α-kafirin	23-25 x 10^3	80	40-90% tert-butanol & reducing agent
	β-kafirin	16-20 x 10^3	7-8	10-60% tert-butanol & reducing agent
	γ-kafirin	28 x 10^3	9-12	10-80% tert-butanol & reducing agent

A summary of the nomenclature for the different solubility fractions of zein and kafirin Table adapted from Shewry (2002); based on the data from Esen (1987), Shull *et al.* (1991) and Shewry (2002)

Kafirin and zein are considered to be very hydrophobic proteins, based on their amino acid composition (Wall & Paulis, 1978). According to Campbell (1999) the amino acids alanine, valine, leucine, isoleucine, proline, phenylalanine, tryptophan, methionine and in some classifications also glycine, are non-polar amino acids because of their non-polar side chains. However, kafirin contains more valine, alanine and isoleucine than zein (Wall & Paulis, 1978; Lásztity, 1984) and is therefore considered more hydrophobic than zein. This may also be the reason for the poorer solubility of kafirin in

70% ethanol (Wall & Paulis, 1978). Duodu, Taylor, Belton & Hamaker (2003) calculated the free energies of hydration of kafirin and zein. Even though the α-prolamins of both kafirin and zein proved to be equally hydrophobic, kafirin was found to be more hydrophobic than zein, because the γ-kafirin was more hydrophobic than the γ-zein. In comparison, kafirin and zein were found to be more hydrophobic than the wheat gluten proteins (Duodu *et al*., 2003). Therefore, based on their hydrophobic characters, zein and even more so kafirin are considered suitable materials for application as a biodegradable plastics or coatings/films.

2.1.2 Extraction of prolamin proteins

Landry & Moureaux (1970) developed a procedure to isolate five protein fractions from maize grain through sequential extraction. This laborious fractionation, using 2-ME in combination with aqueous alcohol, salt and sodium dodecyl sulphate (SDS) as a detergent, made it possible to isolate zein as well as cross-linked zein and other proteins with amino acid compositions close to that of zein.

Taylor *et al.* (1984) extracted total kafirin with 60% *tert*-butanol together with dithiotreitol (DTT) as a reducing agent and compared its extraction efficiency with an extraction done with 70% propan-2-ol and 2-ME. The extraction with 70% propan-2-ol and 2-ME was found to be very temperature dependent in comparison to that of *tert*-butanol plus DDT and the maximum amount of protein extracted was slightly less than the amount extracted with *tert*-butanol plus DTT at room temperature.

Jones & Beckwith (1970) also found the extraction of kafirin with aqueous *tert*-butanol superior to that with ethanol. Kafirin was soluble in 60% *tert*-butanol at room temperature as well as at 60℃, but when extracted with 60% ethanol, the temperature must be increased to 60℃. Wall and Paulis (1978) stated that the reason why sorghum kafirin is less extractable than zein is due to its hydrophobicity.

According to Taylor, Schüssler & Liebenberg (1984), the extraction of maize and sorghum grain done according to the Landry and Moureaux method leads to two prolamin fractions. Upon extraction with aqueous alcohol, classical zein is extracted from maize and kafirin from sorghum. When extracting with aqueous alcohol together with a reducing agent, the zein-2 and crosslinked kafirin is extracted. The zein-2 and crosslinked kafirin is situated in the protein bodies of the starchy endosperm tissue (Taylor *et al.*, 1984). It seems, according to the literature, that these prolamins that need a reducing agent for extraction exist in the endosperm in the form of high molecular weight disulphide (SS)-linked oligomers (El Nour, Peruffo and Curioni, 1998). Duodu, Nunes, Delgadillo, Parker, Mills, Belton & Taylor (2002) found these oligomers, however, to be present in greater quantities in sorghum protein than in maize. In other words, they are also present in zein, but to a lesser extent than in kafirin.

For zein extraction on a commercial scale, a countercurrent extractor was developed in the 1940s for continuous zein extraction from maize gluten meal with aqueous propan-2-ol instead of aqueous ethanol (Lawton, 2002). Propan-2-ol has the advantage of being able to be used without a government license. Through the years, the process has been modified, for example an added alkali treatment has been included which causes the extracted zein to be more stable against gelation as well as in solution. The current method most often used for zein extraction, is the method patented by Carter & Reck in 1970 (Lawton, 2002). According to this process, zein is extracted with 60-90% aqueous propan-2-ol or ethanol, containing sodium hydroxide at temperatures between 55℃ and 70℃ (Carter & Reck, 1970). Kafirin resembles zein in various properties (Shull *et al.*, 1991) but it is less soluble in 70% ethanol than zein (Wall & Paulis, 1978). The method of Carter & Reck (1970) has, however, successfully been used for kafirin extraction from sorghum flour (Da Silva, 2003).

2.2 Cereal protein films

As stated, the use and accumulation of synthetic or non-biodegradable packaging material is one of the main causes for great environmental problems (Marquié, Aymard, Cuq & Guilbert, 1995). Biodegradable packaging, made from natural biopolymers, which are inexpensive and abundant, can be a solution to these problems and cereal protein is an important example of such a biopolymer.

Edible coatings and films can be defined as edible materials that are applied to food surfaces in thin layers either by wrapping, immersing, brushing or spraying, with the intension of increasing the product quality and shelf-life (Gennadios & Weller, 1990). Films are freestanding, thin sheets usually formed separately and applied on a product, whereas coatings are applied directly to the product surface where it is formed. The film or coating acts as a barrier, preventing moisture, oxygen, aroma and/or oil from migrating to and from the food. It also provides protection against potential mechanical damage (Krochta, 2002).

According to Kester & Fennema (1986), edible films can be manufactured from polysaccharides, lipids, proteins as well as composites of these materials. This review will focus on protein films, and mainly on cereal protein films. Edible films and coatings made from cereal proteins, like wheat gluten and maize zein, has had extensive coverage over the years because their relatively hydrophobic nature makes them good moisture barriers (reviewed by Gennadios & Weller, 1990; Aydt, Weller &Testin, 1991; Park *et al.,* 1994; Cuq, Gontard & Guilbert, 1998). Recently, kafirin has been used to produce protein films (Buffo *et al*., 1997; Da Silva, 2003; Taylor, 2003; Emmambux, 2004). These films have great potential due to the many similarities that exist between kafirin and zein.

2.2.1 Film structure and formation

In order to make a film, at least one component with a high molecular weight is necessary to yield a film matrix with cohesive strength (Banker, 1966). Cohesion is the ability of a material to form strong bonds on a molecular level within itself, which will prevent separation at the point of contact. Proteins are ideal for this purpose, being natural polymers, based on amino acids that are linked together by peptide bonds (Hoseney, 1994). The molecular weight of proteins can vary from a few thousand to several million. Proteins are able to form three-dimensional structures. These structures are mainly stabilized through non-covalent interactions (Cuq *et al.*, 1998) for example Van der Waals forces, hydrogen bonding, electrostatic, hydrophobic or disulphide cross-link interactions between the amino acid units (Krochta, 2002).

Film formation from cereal proteins can be made easier to understand when one considers a model for protein structure, for example that for zein proposed by Argos, Pederson, Marks & Larkins (1982) (Fig. 1). This model was constructed through structure prediction based on the results obtained from circular dichroism. Alpha-zein is considered to consist of cylindrical polypeptide capsules, each containing 9 adjacent α-helices. Each helix is made up out of 20 amino acids, of which a few are polar and several are hydrophobic. At the ends of each capsule, a repeat sequence of glutamine residues can be found, and here the capsules are joined together through hydrogen bonding. Among the polar and hydrophobic amino acids in the different α-helices, intra- and intermolecular hydrogen bonds and Van der Waals forces exist, causing the zein capsules to aggregate. Since zein and kafirin are similar in structure and amino acid composition (Shull *et al*, 1991), this model can also be taken as a possible description for kafirin molecular structure.

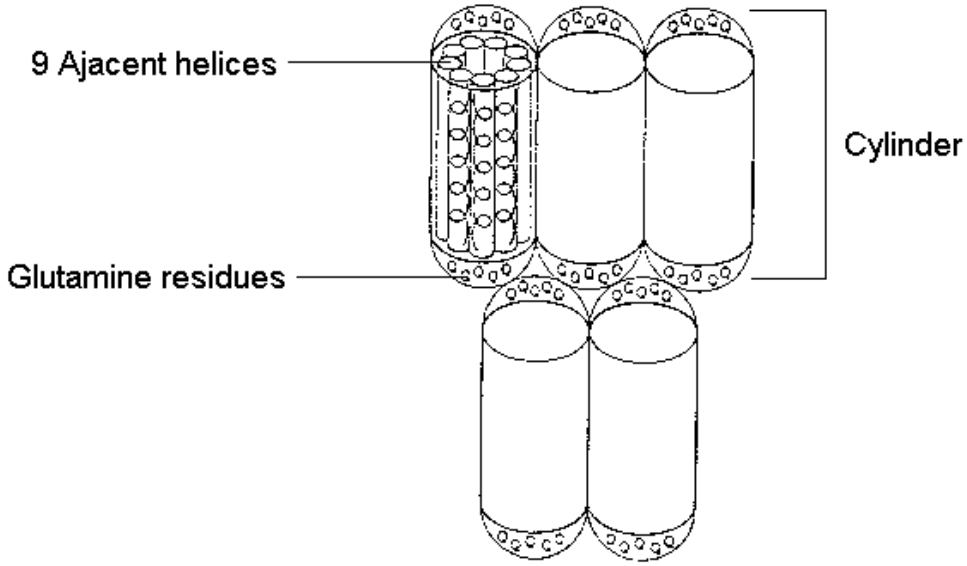


Figure 1: A possible model to show the zein protein arrangement within different planes (Argos *et al*., 1982)

Either a wet process, based on the solubilization, can form films from proteins or dispersion of proteins or a dry process, which is based on the thermoplastic properties of proteins at very low water concentrations (Cuq *et al*, 1998). The wet process is a process by which the protein is dissolved in solvents, commonly water or aqueous alcohol (Krochta, 2002). In some cases the temperature needs to be raised in order for the protein to dissolve, e.g. kafirin dissolves in 70% ethanol only at 60°C (Hoseney, 1994). The film is formed when the prepared formulation is poured or applied to the casting surface, after which the solvent is allowed to evaporate (Krochta, 2002). Proteins are separated from the solvent through precipitation or phase changes (Cuq *et al*, 1998). When the solvent is removed, the film will form due to an increase in the polymer concentration. This will induce bond formation, which leads to the formation of a three-dimensional structure.

The dry process for making protein films is done by thermal or thermo-mechanical processes under low-moisture conditions. The melted protein-based material is then shaped by extrusion, roller milling or thermo moulding

(Cuq *et al*, 1998). Lai, Padua & Wei (1997) made use of this process when preparing zein resin films by melting zein in a microwave, followed by kneading and rolling to form zein sheets.

As stated, films, made from cereal proteins are very brittle, due to extensive intermolecular forces that exist between the polymer chains (Banker, 1966). This brittleness serves as a limitation to the commercial application of the films. In order to overcome this, a plasticizer must be added to improve the functional properties of the films. Plasticizers can be defined as substances that change certain physical and mechanical characteristics of the material they are added to, by reducing the intermolecular forces between polymer chains (Banker, 1966).

2.2.2 Mechanical properties

For comparison of different films with one another, measuring the mechanical properties of these films is a good starting point. Mechanical characterization of films will give important information on how films will react in a specific application, for instance impact strength, film stability under different temperature changes, as well as other environmental and physical stresses (Banker, 1966). Film attributes, like tensile strength (TS), elongation (E) and the modulus of elasticity (EM), also known as Young's modulus are parameters used to characterize the films (Banker, 1966; Krochta 2002).

Tensile strength can be defined as the maximum tensile stress that will develop in a test specimen before it breaks when a tensile test is performed under prescribed conditions (American Society for Testing and Materials, 1986). Tensile stress is expressed as force per unit cross-sectional area. Elongation is the degree to which the film can stretch before it breaks (Krochta, 2002). The % strain for each film is calculated by dividing the elongation of the film strip at break by the original film strip length, multiplying by 100 (Aydt *et al.,* 1991). This would demonstrate the relative deformation in length as a reaction of a specimen to a tensile force (American Society for Testing and Materials, 1986). The modulus of elasticity is a measurement of

the film stiffness, in other words the ability of the film to resist great stress while undergoing very little elastic deformation (Banker, 1966). These stress-strain properties of films can be determined by measuring the linear expansion of filmstrips under an increasing force and can be illustrated with the following figure.

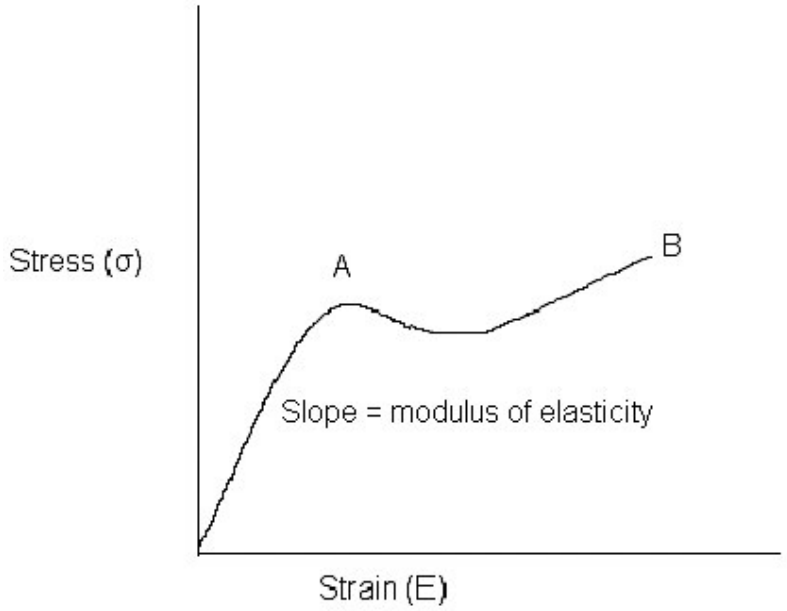


Figure 2: A stress-strain curve for a polymeric film (Banker, 1966)

On the stress-strain curve in Figure 2, the slope of the straight-line portion gives the modulus of elasticity for a tested sample (as reviewed by Banker, 1966). Stronger or stiffer films will have a greater slope, meaning that they have a higher modulus of elasticity. Therefore, more stress will be required to bring about a certain amount of deformation. Point A of the stress-strain curve represents permanent deformation. Here, the film undergoes a marked increase in strain for the first time, without a corresponding increase in stress. Point B represents the breaking point of the film. With some materials, point A on the graph might be absent because they break before they deform. The distance between point A and B shows the degree of plastic deformation that takes place before the sample breaks (as reviewed by Banker, 1966).

With the addition of a plasticizer, the film brittleness will be reduced and the flexibility will increase. The theory to explain these changes imparted by the plasticizer is that it decreases the intermolecular forces along the polymer chains. In other words, it causes a reduction in cohesion (as reviewed by Banker, 1966). The film properties will be drastically influenced by the level of added plasticizer (Krochta, 2002). The TS and EM will decrease with increasing plasticizer content. The E of the film will increase with increasing plasticizer content. It should be noted that factors like film thickness, method of preparation, testing speed, the type of grips that are used during testing, as well as the way of measuring the extension will also have an influence on the measured tensile properties (American Society for Testing and Materials, 1997).

2.2.3 Barrier properties

When considering materials for food packaging uses, their ability to act as moisture barriers is very important because this is required in many applications, especially food applications (Lai & Padua, 1998). The moisture and gas barrier properties of the kafirin films will, for example, improve the average shelf life of fruits with a minimum of 25% as well as reduce the oxidation rate of peanuts with as much as 50% (Enviropak, 2004). Water vapour permeability (WVP) is the time rate of water vapour transmission through unit area of flat material of unit thickness, under specified temperature and humidity conditions, which is induced by the vapour pressure difference between two specific surfaces (American Society for Testing and Materials, 1997). Water vapour transmission rate (WVTR) is the steady flow of water vapour in unit time through unit area of a material, normal to specific parallel surfaces, under specified conditions of temperature and humidity at each surface (American Society for Testing and Materials, 1997).

When compared to edible waxes, often used as moisture barrier coatings on products such as fruit, vegetables, confectionary and drugs, the WVP of protein films is reasonably high (Krochta, 2002). The aqueous alcohol-soluble cereal proteins, zein and gluten, seem to produce films that have a low WVP

in comparison to other film forming proteins, except for fish myofibrillar proteins, which also form films with a low WVP (Krochta, 2002). The low WVP for zein and gluten is because the prolamins are hydrophobic proteins (Esen, 1987). Since, as discussed, kafirin is somewhat more hydrophobic than zein (Duodu *et al.*, 2003), it should therefore have even better water vapour barrier properties.

Plasticizers not only have an effect on the tensile properties of films, they also influence the barrier properties of films. Gontard, Guilbert & Cuq, (1993) used glycerol as a plasticizer in wheat gluten films and found that the WVTR of the plasticized film increased with glycerol content. Park *et al.* (1994) also found that the WVP of both zein and gluten films increased with an increasing plasticizer concentration. It was also seen that the increase in WVP of the films containing only glycerol was greater than films containing a combination of glycerol (very hydrophilic) and polyethylene glycol (less hydrophilic). As already mentioned, it is hypothesised that plasticizers reduce the intermolecular forces between polymer chains (Banker, 1966). The increase in permeability may be due to these modifications in the film structure, brought about by the plasticizers (Gontard *et al*, 1993). The protein network might be less dense, increasing the permeability to gasses and vapours. The hydrophilic nature of glycerol will also favour the adsorption and desorption of water molecules (Gontard *et al.* 1993).

Protein films in general also have low oxygen permeability (OP) at low to intermediate relative humidities (Krochta, 2002). Protein films will therefore be applicable in situations where a low OP is required, for instance as coatings or pouches to suppress aerobic respiration of fresh produce in order to prolong its shelf life (Kester & Fennema, 1986). According to Park *et al.* (1994), plasticizer concentration also has an effect on the OP of cereal protein films. It was found that an increasing amount of glycerol in gluten and zein films led to an increase in the OP of these films (Park, Weller, Vergano & Testin (1992) as referred to by Park *et al.*, 1994). This supports the statement made by Gontard *et al.* (1993) that plasticizers make the protein network less dense, enabling gasses and vapours to pass through more easily.

2.2.4 Influence of environmental conditions on film properties

The characteristics of films such as tensile and barrier properties are affected to a great extent by environmental factors like relative humidity (RH) (Gennadios, Park & Weller, 1993) and temperature (Gennadios *et al.*, 1993; Yoshino, Isobe & Maekawa, 2002). Other factors, though not environmental, that can have an influence on film characteristics, are the casting solvent (Yamada, Takahashi & Noguchi, 1995) and film orientation (Yoshino *et al.*, 2002).

The RH plays an important role in influencing film properties, because water can act as a plasticizer (Gontard *et al.*, 1993). Water is a low molecular weight component, and by reducing the interactions between the protein chains as a plasticizer does (Banker, 1966), it can increase the free volume between the chains, enhancing molecular movement through the film structure (Gontard *et al.*, 1993). This will lead to a reduction in the barrier properties of the film. Water can also influence film tensile properties. Gennadios *et al.* (1993) found that the RH of the surroundings has an effect on film tensile properties. Films were cast from zein and gluten, as well as from two types of cellulosic materials, methylcellulose and hydroxypropyl cellulose. The films were plasticized with glycerol. It was found that with an increasing relative humidity (23% to 75%), the tensile strength of all four film types decreased. It was concluded that this was due to an increase in the moisture content of the films with rising RH (Gennadios *et al.*, 1993). As stated earlier, plasticizers decrease the tensile strength of films (Krochta, 2002), and in this case, the absorbed water acted as the plasticizer. Even though cereal prolamins are hydrophobic proteins, most plasticizers used in these types of films, like glycerol and polyethylene glycol are very hydrophilic (Park *et al.*, 1994). The physical properties of the plasticizer used in the film may affect the mechanical properties of the film, for example, if a plasticizer is very hygroscopic, it may increase moisture uptake by the film (Banker, 1966), again influencing the film's tensile and barrier properties.

Temperature is another environmental factor that may affect film properties to a great extent. Yoshino *et al.* (2002) examined the effect of different preparation conditions on the physical properties of zein films. Films were dried at temperatures, ranging from 30℃ to 45℃. It was found that the drying conditions of the zein films, which had been cast with aqueous ethanol, had a great influence on the tensile strength of the films. The tensile strength increased with increasing drying rate, in other words with a higher temperature (45℃) and a low RH (5%). Films with a slower drying rate (35℃ and 90% RH) showed a low tensile strength (Yoshino *et al.*, 2002).

2.3 Plasticizers

As stated, a plasticizer is a substance which, when added to other materials, changes certain physical and mechanical properties of those materials (Banker, 1966). Plasticizers are generally nonvolatile, nonseparating substances with a high boiling point. Plasticizers are therefore used to either help in processing or to modify the properties of the final product (Sears & Darby, 1982).

As stated, protein films and coatings are in general very brittle. This is due to widespread interactions that exist between protein chains, such as hydrogen bonds, hydrophobic bonds, electrostatic forces and/or disulphide cross-linking (Krochta, 2002). These forces act between the protein chains and hold them together. It is theorized that the added plasticizer will decrease these intermolecular forces along the polymer chains, causing a reduction in cohesion (Banker, 1966). The plasticizer, a small molecular, hydrophilic substance, will compete with the polypeptide to form hydrogen bonds or electrostatic interactions with the protein chains (Krochta, 2002). The addition of a plasticizer to a polymer leads to a decrease in the tensile strength, a lower softening temperature and a decrease in the glass transition temperature (T_g) (as reviewed by Banker, 1966). The biggest effect of the plasticizer is the lowering of the T_g of the substance to below room temperature, changing it from a hard, brittle, glass-like substance to a soft, flexible material at room temperature. As discussed, the addition of a

plasticizer will also have an effect on the barrier properties of the polymeric film, for example the gas and water vapour barrier properties.

Plasticization can be divided into two types: external plasticization and internal plasticization (as reviewed by Banker, 1966). External plasticization is when a substance is added to the polymer it is meant to plasticize, and it may be physico-chemically associated to it. This leads to a reduction in structural cohesion and will cause the polymer structure to be softer and more extendable. External plasticization allows one to regulate the degree of flexibility of the plasticized material according to the type and amount of plasticizer added (Sommer, 1985). Internal plasticization is when similar changes in polymer structure are brought about by changing the internal chemical structure of the polymer, for example through co-polymerization (Banker, 1966). This means that the monomers of homopolymers with a high T_g are co-polymerized with the monomers of which the homopolymers have a lower T_g (Sommer, 1985). The advantage of internal plasticization is that it leads to very stable chemical linking between rigid and flexible segments, but the technology and costs associated with it, limits its application. During this research on plasticization of kafirin films, use will be made of external plasticization only.

There has been much theorisation on how exactly plasticizers work. Entwistle & Rowe (1979) state that an effective plasticizer must be able to interact with the forces holding the polymer chains together by positioning itself between the chains. Cuq, Gontard, Cuq & Guilbert (1997) state that low molecular weight plasticizers are able to fit between protein chains, where they form hydrogen bonds with the reactive groups of the proteins, forming protein-plasticizer bonds instead of the protein-protein bonds. Lai & Padua (1998) state that a plasticizer acts as a spacer between the polymer chains, decreasing the intermolecular forces between them, which cause the polymer to be more flexible. The theories will now be discussed in more detail.

2.3.1 Theories on plasticization

People have tried to explain plasticizer action for years and three main theories have been proposed to account for the effect plasticizers have on polymers. They are the lubricity theory, the gel theory and the free volume theory (Sears & Darby, 1982). These theories are very complex and the best possible attempt to explain them is by paraphrasing what Sears & Darby (1982) wrote.

2.3.1.1 The Lubricity Theory

The resistance of a brittle polymeric substance to deformation, in other words, its rigidity, is because of the intermolecular friction that exists between the macromolecules. As a polymer is flexed or deformed, the macromolecules work back and forth over each other on internal glide planes. In order to make this back and forth movement of the resin macromolecules easier, a plasticizer is added to lubricate these internal glide plains, acting as oil between two moving parts. The plasticizer therefore gives internal lubricity.

With this theory, it is assumed that no bonding between macromolecules exists, except for where surface irregularities occur. It is assumed that there is only, if any at all, very weak bonding between the plasticizer molecules and/or the plasticizer molecules and the polymer molecules. This weak bonding could be described as similar to the low interfacial energy that exists between a solid and a liquid lubricant.

The lubricity theory is not really used any more today as the only theory of plasticization, but when used in conjunction with other theories it can be handy to explain certain phenomena associated with plasticization.

2.3.1.2 The Gel Theory

According to the gel theory, the resistance of a resin to deformation is due to an internal, three-dimensional honeycomb structure. It states that a gel is

constructed out of loose attachments occurring along the polymer chains at certain intervals. For a stiff or brittle material, the attachments will occur closer to each other, causing the cell dimensions to be small. Such a material will not be elastic because it cannot put up with attempts to deform it. In the opposite case, where the points of attachment between macromolecules are widely separated, the material will be flexible without a plasticizer.

Therefore, when a plasticizer is added to a resin with many attachments along its polymer chains, the plasticizer will break the attachments at some of the places. As already mentioned, polymer chains are held together through different forces. After breaking the attachments, the plasticizer will mask the centres of the forces by selectively solvating the polymer chains at these points, and this results in the same effect as having less points of attachment on the macromolecules. This will potentially result in a less rigid gel structure.

Newer and modern concepts to explain plasticizer action take this theory into account, but it is not seen as sufficient to explain why flexibility is brought about in a polymer by a plasticizer. This is because there are free plasticizer molecules present in a polymer system, which are not attached to polymers directly. They are indirectly attached through other plasticizer molecules. These unattached molecules also increase flexibility because they appear to be effective in swelling the gel and they facilitate molecular movement in the polymer. Through this, they increase the flexibility of the polymer and they may actually constitute the greatest volume of the plasticizer. It is therefore also necessary to make provision for this, and the gel theory does not.

2.3.1.3 The Free Volume Theory

The development of the lubricity and gel theories was a rapid process because these theories were derived from easily visible structures and simple analogies. To explain plasticization by means of the free volume theory took a little more time since this theory grew out of the less evident characteristics of materials like crystals, glasses and liquids. This theory depends very strongly on mathematical corroboration for is validity and strength. For the application

of this theory, it is crucial to have a clear understanding of what the glass transition temperature, T_g, of a substance is. This is because one of the major functions of a plasticizer is to lower the T_g of the polymer.

To explain the free volume concept, an example of an ideal crystal versus a real crystal should be visualized. We imagine that the atoms or molecules of an ideal crystal are set perfectly compact in a completed lattice at absolute zero. This is not the case for a real crystal though. In a real crystal the atoms or molecules are not as compact as imagined. The reason for this might be because they vibrate in a non-harmonic fashion or due to imperfections that may exist in the lattice structure they are set in. These imperfections or spaces are called holes. In comparison to a crystal, the amount of holes in a liquid are considerably greater than in a crystal. As a result, the free volume can be considered as two parts: (1) a continuous part, which is present as a result of persisting oscillations and increases only slightly with the rising of temperature and (2) a discontinuous part called holes which increases to a great extent when the temperature rises.

An increase in free volume will cause an increase in the movement of the polymer molecules, and because of this, the study of plasticization was called “the study of ways to increase free volume”. There are three main sources for free volume in a polymeric chain. They are the motion of the chain ends, the motion of the side chains and the motion of the main chain. To increase these motions, which lead to an increase in free volume of a resin system, certain actions can be taken:

- The number of end groups of the polymer chains could be increased.
- The number or the length of the side chains could be increased (internal plasticization).
- Sections with low stearic hindrance and little intermolecular attraction, for example low polarity and hydrogen bonding could be included. This will increase the chance of main chain movement, increasing the free volume (internal plasticization).

- The inclusion of a compound with a lower molecular weight, which will increase the free volume through the three actions above (external plasticization).
- The temperature could be raised through the T_g (plastication).

2.3.2 Glass transition

The addition of a plasticizer to a polymer will lead to the lowering of the T_g of the polymer and this phenomenon can be explained by means of the free volume theory (Sears & Darby, 1982).

The definition of glass transition is the conversion of a brittle glass to a highly viscous or rubbery solid (Gontard *et al.,* 1993). Above the T_g of a polymer, the glassy state will change to a rubbery state where the molecules of the material will have enough energy to move. This molecular motion produces a great amount of free volume, which is called the "hole free volume" (Sears & Darby, 1982). The T_g of a material is a function of its chain mobility and the purpose of a plasticizer is to increase the chain mobility of the material (Entwistle & Rowe, 1979). The effect the plasticizer has on the T_g of a polymer is therefore a specific measure of how efficient the plasticizer is.

According to Sears & Darby (1982) plasticizer molecules are usually much smaller than the polymer molecules. For example, in this particular case, α-kafirin has a M_r 23-25 x 10^3 (Shull *et al.* 1991) in comparison to the molecular weight of a plasticizer such as glycerol (92) (Park *et al.* 1994). Therefore, plasticizers contribute a greater free volume for each molecule per total volume of material because they possess a larger portion of end groups in comparison to the polymer and their T_g is much lower than that of the polymer (Sears & Darby, 1982).

Sears & Darby (1982) reviewed an equation, developed by Kanig in the 1960s, for comparing the efficiency of different plasticizers in reducing T_g. Out of this equation, a few predictions were made:

- A plasticizer with a small molecular size would be more effective in lowering the T_g of a polymer (Sears & Darby, 1982).

This was proved by Di Gioia & Guilbert (1999) who found that the depression of the T_g of maize gluten meal-plasticizer blends was greater for smaller molecular size plasticizers at equal volume fractions. The small molecular size plasticizers they used, were water (MW 18) and glycerol (MW 92). They were found to have a greater plasticizing effect than plasticizers with larger molecular weights, such as palmitic acid (MW 256) and dibutyl tartrate (MW 262).

- Plasticizer efficiency is proportional to the T_g of the plasticized polymer, which means that if the resin has a high T_g, the efficiency of the plasticizer will be greater. Therefore, the first small amounts of added plasticizer will be the most effective in lowering a high T_g and it will become less effective as the plasticizer amount increases and the T_g lowers (Sears & Darby, 1982).

Again, Di Gioia & Guilbert (1999) found this to be true, observing that the depression of the T_g of maize gluten meal films, plasticized with various plasticizers, was the greatest for the first small amounts of all the plasticizers that were added. As the amount of added plasticizer increased, the depression of the T_g became less effective.

- The plasticizer will be much more effective when the affinity between the plasticizer and the polymer is small in comparison to the polymer-polymer affinity. In other words, if a substance is a good plasticizer to a specific material, it would be a bad solvent to it (Sears & Darby, 1982).

- Plasticizers are most efficient if the plasticizer-plasticizer affinity is weak in comparison to polymer-polymer affinity (Sears & Darby, 1982).

It is important to know that these conclusions are valid and useful, but there can be exceptions to the rule. Therefore, when choosing a plasticizer, it is necessary not only to focus on these conclusions but to take into account other factors too, for example the volatility and exudation of the plasticizer (as reviewed by Sears & Darby, 1982).

2.4 Possible plasticizers for cereal protein films

It is clear that cereal protein films need plasticizers. Without plasticizers, they are too brittle to be used as free standing films. To increase film flexibility, a plasticizer or a combination of plasticizers must be added. As stated, it is hypothesised that the plasticizer brings about an increase in flexibility by reducing or decreasing the intermolecular forces that occur along the polymer chains (Banker, 1966). The increase in flexibility is accompanied by a reduction in film strength. For successful plasticization of a polymer system, the plasticizer and the polymer must be compatible, in other words miscible. This will indicate similar intermolecular forces in the two components. It is also ideal if the plasticizer is readily soluble in the solvent that is used for film preparation, which will prevent separation from taking place too early during drying (Banker, 1966).

During this discussion, plasticizers will be considered, which in the literature have already been experimented with in the production of films from various proteins, and appear to have potential for kafirin film making.

2.4.1 Polyhydric alcohols (polyols)

Sugar alcohols or alditols are also known as polyhydroxy alcohols, polyhydric alcohols or polyols (BeMiller & Whistler, 1996). Polyhydric alcohols are derivatives of carbohydrates and are usually used for their low-calorie

sweetener properties (Lindsay, 1996). They contain only hydroxyl groups as functional groups, and therefore they are generally water-soluble and hygroscopic. Polyols include substances like glycerol, sorbitol, xylitol, propylene glycol as well as polymeric forms of polyhydric alcohols, such as polyethylene glycols (Lindsay, 1996). Polyols are effective plasticizers because they are able to reduce internal hydrogen bonding between molecules, increasing the spacing between the molecules of the substance they are added to (Kim, Marx, Weller & Hanna, 2003). Polyols have been found to be especially effective in the plasticization of hydrophilic polymers (Cuq *et al.*, 1997). Polyols, which are often used to plasticize protein films, are glycerol, sorbitol and polyethylene glycol and these will be discussed in detail.

2.4.1.1 Glycerol (G)

G is a hydrophilic polyol (Lindsay, 1996). It is a relatively small molecule, with 3 carbons per carbon chain and a molecular weight of 92. Gontard *et al.* (1993) used G to plasticize wheat gluten films. G plasticizer action was attributed to it being easily inserted between protein chains due to its small size. Inserted, it will reduce the protein-protein interactions and form hydrogen bonds with the amide groups of the proteins. This reduces the closeness between the protein chains, making the protein structure less dense. According to Cuq *et al.* (1997), it is with the incorporation of plasticizers and therefore the reduction in the density of the intermolecular interactions, that more free volume is created. This contributes to the plasticizing effect.

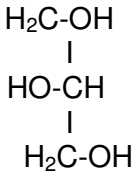


Figure 3: The chemical structure of glycerol (Crosby, 1992)

When G was used as a plasticizer in wheat gluten films, an increase in water vapour transmission rate through the films was observed as the G content of the films increased (Gontard *et al.*, 1993). The explanation given for this was that when G infiltrated the three-dimensional protein network, it brought about a structural modification to the protein network, which made the network less dense. This made it easier for water to be transferred across the film. Another reason for the increase in water vapour transmission could have been that the adsorption and desorption of water molecules was favoured by the hydrophilic nature of G (Gontard *et al.*, 1993).

Migration of G in protein films is a problem. According to Park *et al.* (1994), Park, Weller, Vergano & Testin (1992) found that G tends to migrate from the protein film solution to the surface of the film when it is used alone as a plasticizer. Signs of G migration were when the surfaces of new, transparent maize zein films turned greasy within a few hours after production as well as when films lost their flexibility over time (Park *et al.*, 1992, as reviewed by Park *et al.*, 1994). The migration was attributed to limited binding between the protein molecules and G. This could be an explanation, but is somewhat contradictory to what was earlier said about G's plasticization mechanism as according to Gontard *et al.* (1993) and Cuq *et al.* (1997). There are factors that will influence the speed of G migration, such as the functional groups, the polarity and the structure of the film matrix (Park *et al.*, 1992, as reviewed by Park *et al.*, 1994).

Even though the addition of G improved the flexibility of gluten films, Gontard *et al.* (1993) stated that the amount of G added to a film should be limited due to the effect it has on the film strength and the water vapour barrier properties. With the increasing G amount, the tensile strength of the gluten films decreased and the film became more permeable to water vapour. Working with zein films, Lawton (2004) found G to be a very hygroscopic plasticizer. Films containing hygroscopic plasticizers tend to absorb a lot of water from the atmosphere, which will have additional effects on the tensile properties of the films.

2.4.1.2 Sorbitol

Sorbitol is also a hydrophilic polyol with a low molecular weight (182), but almost twice that of G. Because of its small molecular size, its molecules can also easily fit between protein chains to form hydrogen bonds with the reactive groups of the proteins (Cuq *et al.,* 1997). Sorbitol has often been used as a plasticizer in a variety of different protein films, for example in gluten films (Cherian, Gennadios, Weller & Chinachoti, 1995), films made of fish myofibrillar proteins (Cuq *et al.*, 1997), soy protein films (Cho & Rhee, 2002) as well as whey protein isolate films (Shaw, Monahan, O'Riordan & O'Sullivan, 2002). Cuq *et al.* (1997) used sorbitol, glycerol and sucrose as plasticizers for fish myofibrillar protein films at equal molecular contents and no apparent differences were picked up in the mechanical properties for each of the different films. However, G was found to have a slightly higher plasticizing effect than sorbitol and sucrose at the highest amount used. The reason was said to be that G's smaller molecular size made it easier to be inserted within the three-dimensional protein network.

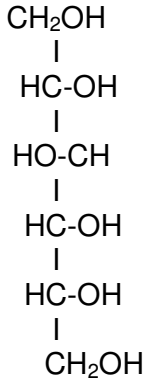


Figure 4: The chemical structure of D-glucitol (sorbitol) (BeMiller & Whistler, 1996)

2.4.1.3 Polyethylene glycol (PEG)

Polyethylene glycol is a substance, much larger in molecular weight than G and also less hydrophilic (Park *et al.*, 1994). According to Lindsay (1996), a

polyol is a substance that contains only hydroxyl groups as functional groups, and that PEG can be classified as a polyol. From the structure in Figure 5, the end groups of the molecule are not visible, but in the basic PEG structure no hydroxyl groups are present to clearly indicate that PEG is a polyol. This is therefore uncertain. There are different types of PEG, all differing in molecular size and the ones most often used for protein film plasticization are PEG 300, used in zein films (Lawton, 2004); PEG 400, used in gluten and zein films (Park *et al.*, 1994; Park & Chinnan, 1995) and in sodium caseinate films (Siew, Heilmann, Easteal & Cooney, 1999); PEG 600 in maize gluten meal films (Di Gioia, Cuq & Guilbert, 1998) and PEG 1000 in zein films (Tillekeratne & Easteal, 2000). In comparison to the molecular weight of G (92), it is clear that the PEG molecule is in general much bigger in size.

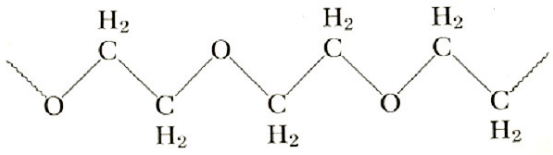


Figure 5: The chemical structure of polyethylene glycol (Lee, Stoffey & Neville, 1967)

Because the PEG molecule is so much bigger than G, it will not diffuse through the film matrix as rapidly (Park e*t al.*, 1994). However, PEG should be much less effective in disrupting protein-protein interactions between protein chains due to its longer chain length (Siew *et al.*, 1999). Because of its larger size, it may be prevented from interacting with some of the hydrophilic sites on a protein chain. It cannot be inserted between protein chains because of stearic hindrance that might exist from adjacent side chains. Therefore, PEG is more likely to cross-link protein chains, promoting hydrophobic intermolecular bonding between them (Siew *et al.*, 1999)

When PEG was used in combination with G to plasticize gluten and maize protein films, opposite trends in the elongation of the two types of films were observed (Park *et al.*, 1994). The elongation of zein films increased with a

decreasing ratio of G/PEG, whereas the elongation of the gluten films decreased. A possible reason for this was said to be the difference in hydrophobicity between gluten and zein. The more hydrophobic a protein, the better the linkages that will form through hydrophobic interactions between the protein molecules and PEG. Since zein is more hydrophobic than gluten (Duodu e*t al.*, 2003), PEG could interact better with zein molecules. This will increase the elongation of the protein film.

The water vapour permeability of both zein and gluten films was found to decrease with a decreasing ratio of G to PEG (Park *et al.*, 1994). This is because PEG is less hydrophilic than G due to its functional groups.

2.4.2 Lipids and derivatives

One of the greatest limitations of applying edible films to food is their inability to prevent moisture loss due to their hydrophilic nature (Park, Testin, Vergano, Park & Weller, 1996). This is true especially for films made from proteins, having a high water vapour permeability (WVP) in comparison to materials like edible waxes or low-density polyethylene (LDPE) (Krochta, 2002). A possible solution to this problem, in other words to improve the barrier properties of these films, is to add a hydrophobic plasticizer like a fatty acid during film production (Lai & Padua, 1998; Pommet, Redl, Morel & Guilbert, 2003).

2.4.2.1 Fatty acids

Oleic acid, stearic acid and palmitic acid are the fatty acids most often used in the literature as additions to cast and resin films, either as plasticizers, for example in zein films (Lai & Padua, 1997; Lai *et al.*, 1997; Lai & Padua, 1998; Santosa & Padua, 2000) and in soy protein isolate films (Lodha & Netravali, 2005), or as protective layers on cast films, for example methylcellulose films, laminated with a zein-fatty acid blend (Park *et al.*, 1996).

Oleic acid (C18:1) is a monounsaturated fatty acid with a melting point of 18.9°C (Stauffer, 1996). Lai & Padua (1998) prepared films from casting zein-oleic acid emulsions as well as by stretching zein-oleic acid resins. It was found that the WVP of the cast films differed between the smooth and the dull side of the film. When the dull side, considered to contain the highest concentration of lipid (Lai & Padua, 1997), was adjacent to a salt solution, the WVP was lower than with the smooth surface towards the solution. Apart from this, the resin films showed a lower WVP than the cast films, indicating that the preparation method also had a great influence on film characteristics (Lai & Padua, 1998).

A model was created by Lai, Geil & Padua (1999) through the use of X-ray scattering, for the structure of zein resin films plasticized with oleic acid. The proposed model (Fig. 5) was based on the dimensions of nonreduced α-zein aggregate. With this model, it was assumed that platelets with a thickness of 135 Å are formed, consisting of pairs of the zein aggregates alternating with oleic acid bilayers, which are bound together through hydrophobic bonding. The aggregates are presumed to be in rows. Each row is 2 aggregates thick and the aggregates of neighbouring rows lie side by side, either aligned or staggered. The oleic acid molecules are illustrated the way they are, due to the *cis* “kink” that exists where the double bond is present.

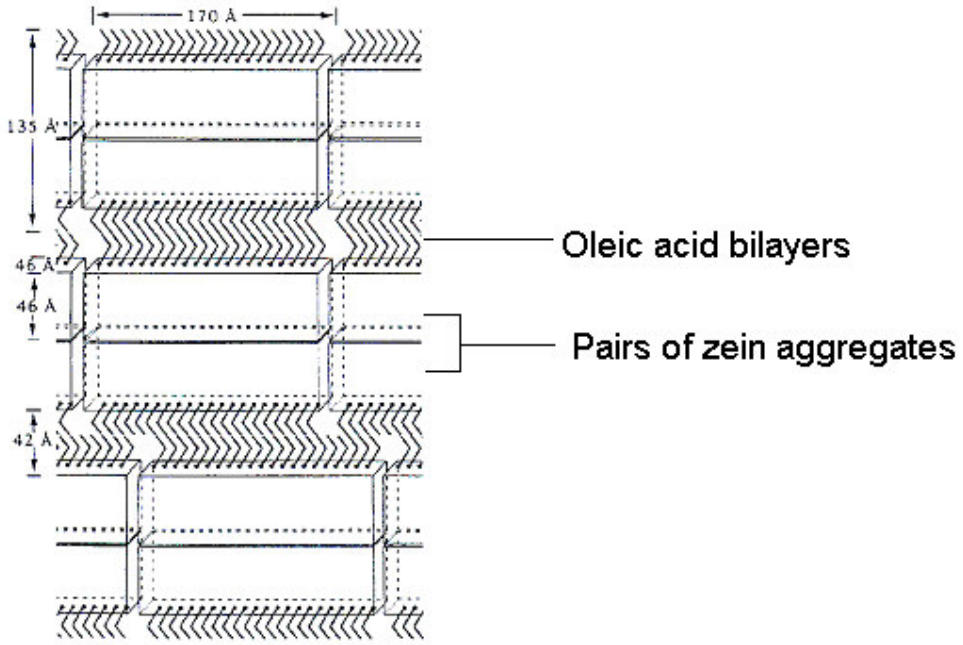


Figure 6: Model proposed for the structure of zein-oleic acid resin films, with the film thickness in the vertical direction (Lai *et al.*, 1999)

Stearic acid is a saturated fatty acid (C18:0) with a melting point of 69.9℃ (Stauffer, 1996). Lodha & Netravali (2005) combined stearic acid with G as plasticizers for soy protein isolate (SPI) based films, because G alone has a tendency to leach out over time. The objective was to see what affect the added stearic acid had on the tensile and the thermal properties of the films, as well as on its moisture absorption. As the stearic acid content of the films increased, with the G remaining constant, the measured Young's modulus increased, reaching its maximum value at 25% stearic acid (by weight of the SPI powder). The remaining tensile properties measured, such as the fracture stress and strain, energy at break as well as the moisture content decreased as the amount of stearic acid increased. The long hydrophobic hydrocarbon chain of the stearic acid molecule was considered one possible reason for the reduced moisture content, the increased Young's modulus and the reduced fracture strain.

Lai *et al.* (1997) used stearic acid and palmitic acid to plasticize zein-based resin films in order to investigate the effect each of them had on the

mechanical properties and the water absorption rate of the films. Like stearic acid (C18:0), palmitic acid is a long chain (C16:0), saturated fatty acid (Stauffer, 1996). The addition of palmitic or stearic acid made the zein resins softer and easier to mould (Lai *et al.*, 1997). The tensile strength of the films was increased to a great extent with the addition of palmitic acid to a ratio of 0.5 g per gram of zein and stearic acid to 0.25 g per gram of zein. Mass ratios higher than this caused a decrease in tensile strength. Overall, the elongation of the zein sheets was not very high, but the sheets plasticized with palmitic acid showed a greater elongation than those plasticized with stearic acid. The water absorption of the films decreased as the fatty acid content increased.

2.4.2.2 Surfactants and emulsifiers

A surfactant is a chemical substance that gathers at the interface between two substances that do not mix, lowering the surface tension between them (Stauffer, 1996). A surfactant is amphiphilic in nature. In other words, it contains a hydrophobic part that prefers a non-polar environment and a hydrophilic part that prefers a polar environment.

An emulsifier is a type of surfactant. Sometimes, emulsifiers are called surfactants but all surfactants are not always successful emulsifiers (Stauffer, 1996). An emulsifier is a substance that will lower the interfacial energy between two phases that will not mix, and through this, it will assist in the dispersion of the one phase into the other.

2.4.2.2.1 Sodium dodecyl sulphate (SDS)

Sodium dodecyl sulphate (SDS) is an anionic surfactant and has the power to cause denaturation and dissociation of proteins (Fairley, Monahan, German & Krochta, 1996). SDS will cause the disruption of hydrophobic bonds between protein molecules or it will prevent them from forming, but it will not split covalent bonds (Fukushima & Van Buuren, 1970).

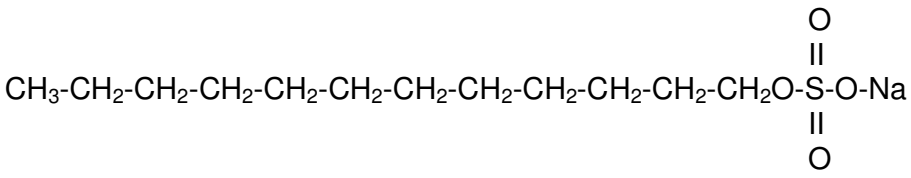


Figure 7: The chemical structure of sodium dodecyl sulphate (SDS) (Merck, 2001)

Rim, Gennadios, Weller & Hanna (2002) used SDS in the preparation of cast films from SPI. The tensile strength of the films was reduced by the addition of SDS. It was said that the formation of strong protein structures through hydrophobic interactions were prevented by the SDS. The hydrophobic region of the SDS molecule interacted with the hydrophobic amino acid residues of the proteins. This caused the formation of "weaker" structures between neighbouring protein chains, leading to the lower tensile strength of films. It was also found that film elongation increased with SDS addition. According to Graveland, Bongers & Bosveld (1979), SDS leads to the unfolding of the protein chains, causing them to become "rod-shaped" due to broken hydrophobic bonds. Rim *et al.* (2002) suggested this to be the reason for the increased elongation, which resulted from this more linear protein molecule orientation.

Because of its ability to disrupt hydrogen bonds and to increase the charge repulsion between neighbouring protein chains, Fairley *et al.* (1996) saw potential for anionic surfactants as plasticizers for protein films. Whey protein isolate films were prepared with SDS as co-plasticizer with sorbitol and G. It was found that SDS has good plasticization properties in combination with the mentioned polyols, but is a poor plasticizer on its own. This was attributed to the low moisture content of the films because SDS needs a minimum amount of water to be effective. Also, surfactants do not disturb the attractive interchain forces between the protein chains enough to act as a plasticizer alone (Fairley *et al,* 1996).

2.4.2.2.2 DATEM

DATEM (diacetyl tartaric ester of monoglyceride) (Stauffer, 1996) is an anionic oil in water emulsifier, usually used in the bread industry to improve the handling properties of wheat dough and to increase the loaf volume of bread (Köhler, 2001).

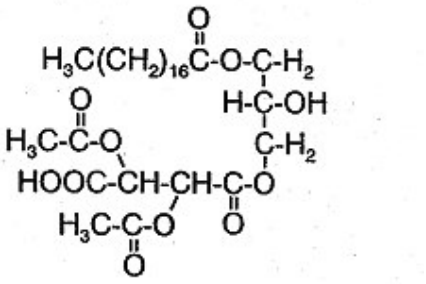


Figure 8: The chemical structure of DATEM (diacetyl tartaric ester of monoglyceride) (Stauffer, 1996)

Di Gioia & Guilbert (1999) used DATEM, octanoic and palmitic acids, dibutyl tartrate and dibutyl phthalate as “amphiphilic” plasticizers and water and G as “polar” plasticizers to plasticize thermoplastic resins based on maize gluten meal. This was done to see what the effect of the different plasticizers was on the T_g of the resins. It was found that dibutyl tartrate and DATEM caused a decrease in T_g equal to octanoic acid, at equal volume fractions. This took place despite the fact that they have very different molecular weights. DATEM (mean MW 630) and dibutyl tartrate (MW 262) are much bigger than octanoic acid (MW 144). This is contradictory to the theory that plasticizers with smaller molecular weights cause a bigger reduction in the T_g of plasticized polymers (Sears & Darby, 1982). Overall, the plasticizing efficiency, in other words the T_g depression, was higher for “amphiphilic” plasticizers than for “polar” plasticizers on a molar basis, DATEM and dibutyl tartrate being much more efficient than octanoic acid, G and water (Di Gioia & Guilbert, 1999).

2.4.3 Acidulants

Acidulants are food additives, usually added to give a tangy or a tart flavour to a product without imparting a characteristic flavour of its own (Liebrand, 1992). Apart from being flavouring agents or flavour enhancers, acidulants and their salts can also be used for other functions in foods, e.g. as antioxidants, curing and pickling agents, as pH control agents, leavening agents etc. Acidulants such as acetic acid, citric acid, lactic acid, tartaric acid, glucono-delta-lactone and many others are often used in the food industry (as reviewed by Berry 2001). Their use in protein film production is, however, a new field.

2.4.3.1 Lactic acid (LA)

Lactic acid is one of the most well-known organic acids, widely found in fermented foods such as yoghurt, cheese, buttermilk, beer, pickles and sauerkraut (as reviewed by Berry 2001). Lactic acid is also known as 2-hydroxypropanoic acid or milk acid. It is a monocarboxylic acid with a molecular weight of 90.

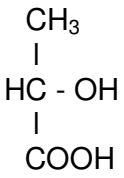


Figure 9: The chemical structure of Lactic acid (Dziezak, 2003)

LA has become an important ingredient in the food industry because of its variety of applications. It can be used for pH reduction. Its mild taste will not mask any other more delicate aromatic flavours. It can be used for flavour enhancement as well as microbial inhibition (Dziezak, 2003).

There is little literature on LA as a plasticizer. Herald, Hachmeister, Huang and Bowers (1996) reported on packaging materials for cooked turkey, which were made from zein. The zein films were plasticized with LA, sorbitol and triethylene glycol. The objectives were to evaluate the mechanical and barrier properties of the films, but mainly to report on the effect of the different additives to reduce oxidative rancidity. It was not focused on the effect off the plasticizers as such. It was reported though, that the plasticizers had an effect in lowering the tensile strength of the zein films.

LA has been reported to be a primary solvent and a good plasticizer for zein films (Lawton, 2002). It was also found to be a good solvent for kafirin (Taylor, 2003). According to Fennema (1996), a good solvent is a good plasticizer, but a plasticizer is not necessarily a good solvent. Therefore, LA is expected to be a good plasticizer for kafirin.

LA does play an important role, together with sulphur dioxide during the steeping step of maize wet milling (Jackson & Shandera, 1995). Long steeping periods are required for efficient separation of proteins and starch in the maize kernel. It lowers the pH inside the kernel, which enhances the effectiveness of the action of the sulphur dioxide, absorbed by the kernel. LA contributes to the separation of the milled components, such as starch and maize gluten (Jackson & Shandera, 1995). Therefore, when using LA in kafirin film production, the lowered pH might have an effect on protein dispersion, optimizing protein solubilization.

2.4.3.2 Glucono-delta-lactone (GDL)

GDL is an acidulant, found naturally in fruits and honey (Dziezak, 2003). GDL is the cyclic 1, 5- intermolecular ester of D-gluconic acid with a molecular weight of 178 (reviewed by Berry, 2001). Commercial production of GDL is by a fermentation process from glucose, making use of the enzymes or pure microbiological cultures such as *Aspergillus niger* or *Acetobacter suboxydans* (Dziezak, 2003). During this process, glucose is oxidized to form gluconic acid, from which GDL is obtained through crystallization. When GDL is added

to water, it will hydrolyze to form a mixture of gluconic acid and its δ- and γ-lactones at equilibrium.

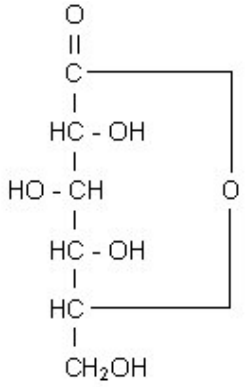


Figure 10: The chemical structure of glucono-delta-lactone (GDL) (Dziezak, 2003)

GDL has a slow rate of acidification and when converting to gluconic acid, the taste will change from sweet to neutral with faint acidic aftertaste (Dziezak, 2003). GDL is therefore a popular acidulant in products like tofu, milk puddings and creamy salad dressings because of its slow acidification rate, its mild taste (neutral with a slight acidic after taste), as well as its metal-chelating properties.

GDL and calcium salts were used by Park, Rhee, Bae & Hettiarachchy (2001) during the formulation of SPI films to determine their effect on the mechanical properties and water vapour permeability of the films. Soy proteins are very hydrophilic and can therefore not be directly compared to films made from hydrophobic kafirin, zein and gluten, but since the literature on the use of GDL in a film application is limited, this is useful information. Park *et al.* (2001) reported that the tensile strength, puncture strength and percentage elongation at break of the SPI films increased with an increasing amount of GDL. It was said that GDL possibly promoted the formation of hydrogen bonds, which contributed to film formation. The addition of GDL also improved the moisture barrier properties of the films. In other words, it lowered the water permeability of the films. By increasing the hydrophobicity and the insolubility of the unfolded proteins, GDL caused them to aggregate and to

form ionic cross-links. The ionic cross-linking would reduce the mobility of the protein segments and the solubility of the proteins in water (Brandenburg, Weller & Testin, 1993). This was the presumed reason for the reduced water vapour permeability through the protein matrix (Park *et al.*, 2001).

From previous experience, GDL was found to have the ability to plasticize kafirin films, leading to changed film characteristics (Personal communication, Ms. S. Buchner, Food Scientist, Bio/Chemtek, CSIR, Pretoria). Based on this, GDL is considered for further investigation.

2.5 Conclusions

There is much information in the literature on protein film plasticization, especially on zein and gluten film plasticization. The plasticization of kafirin films, however, has not been studied thoroughly. Glycerol, polyethylene glycol and lactic acid are considered to be worth an in-depth investigation for kafirin plasticization, based on previous work done in our laboratory on kafirin films (Da Silva, 2003; Taylor, 2003; Emmambux, 2004). In order to assist in protein solvation, SDS and LA are considered good prospects, since SDS is a powerful denaturing agent (Fairley *et al.*, 1996) and LA is a good solvent for kafirin (Taylor, 2003). GDL is considered worth further investigation based on the experience of Ms. S. Buchner (Personal communication) who found it to be a good plasticizer for kafirin films. Emulsifiers such as DATEM are also considered worth investigating in the light of the work done by Di Gioia & Guilbert (1999) on the plasticization of maize zein films.

3. MATERIALS AND METHODS

3.1 Sorghum and flour

Two white condensed tannin-free sorghum cultivars, PANNAR 202 and 606 (ex. Mr. B. Koekemoer, Lichtenburg, 2001) were mixed and decorticated with a carborundum cone abrasive-type rice pearler (Maig Braunschweig, Germany) until an approximate 75% extraction rate (essentially pure endosperm) was reached. The decorticated grain was milled to flour with a hammer mill. The hammer mill was fitted with a sieve, having a mesh opening of 800 µm. The flour was vacuum packed and stored at 7°C to ensure sample stability until further use.

3.2 Extraction of kafirin

Kafirin was extracted from the sorghum flour by following a method based on the solvent extraction process for zein patented by Carter & Reck (1970). The extraction was done with 70% (w/w) ethanol (99.9%), 0.5% (w/w) sodium metabisulphite as a reducing agent and 0.35% (w/w) sodium hydroxide.

The white sorghum flour (500g) was weighed out into a plastic bucket with a tight fitting lid. In the centre of the lid, a small hole was made for the rod of the stirring paddle to go through. Sodium metabisulphite (12.5 g) and sodium hydroxide (8.75 g) were dissolved in distilled water (728.75 g) and mixed with 1750 g ethanol. This was then added to the sorghum flour in the bucket. The bucket with its contents was heated in a warm water bath to 70°C and held at this temperature for 1 hour, while stirring continuously with the stirring paddle. Directly after this period, the slurry was centrifuged for 5 min at 1000 g at room temperature and the resulting supernatant, which contained the extracted kafirin, was decanted off and saved. The spent grain was washed with a further 500 g of extractant and centrifugation was repeated. The recovered supernatants were poured into open trays, covered with muslin cloths to prevent contamination and left overnight in a fume cupboard with the fan switched on for the ethanol to evaporate. After evaporation, only a yellow

curd-like precipitate was left. It was washed into a beaker with a little cold water (< 10℃) and the pH was adjusted to pH 5 with 1 M HCl to neutralize the protein. It was filtered under vacuum, the filtrate was discarded and the residue freeze dried.

After drying, the sample was milled in a coffee grinder to a fine powder. This was defatted by washing it with hexane in a ratio of 10 g kafirin powder: 100 ml hexane. The kafirin and the hexane were poured into a conical flask, covered with aluminum foil to limit evaporation and stirred with a magnetic stirrer bar on a magnetic stirrer plate for 1 hour. This procedure was repeated 3 times, every time decanting the used hexane and replacing it with fresh hexane. After the third time, the hexane was removed by filtration under vacuum and the kafirin was dried overnight at room temperature.

3.3 Casting of films

The defatted kafirin powder (1.44 g pure (100%) protein basis) was weighed into a 100 ml Erlenmeyer flask. As the protein content (N x 6.25) of the kafirin was 87.79% (as is basis), an amount of 1.64 g of kafirin was used. Plasticizer was weighed out (see section 3.3.1) and then 9.0 g aqueous ethanol (70% w/w) was added to it. This was mixed with the kafirin. A magnetic stirrer bar was included and the flask with its contents was weighed. The total weight was recorded before covering the flask with aluminum foil. The flask was put on a stirrer hotplate at 70℃, the stirring motion was turned on slowly to full and it was maintained for 10 min. On top of the flask, a small frozen cooler block was placed to reduce ethanol evaporation. After 10 min had elapsed, the flask was taken from the heat, the foil was removed and it was reweighed. Absolute ethanol was added to replace the evaporated ethanol until the original weight was obtained. The contents were mixed by shaking the flask. Aliquots of 4 g were weighed out into 86 mm internal diameter plastic petri dishes. The petri dish was swirled around very gently to distribute the contents evenly on the bottom. The contents of the flask were enough to prepare 2 petri dishes with 4 g aliquots each. The petri dishes without lids were placed on a level surface (confirmed with a spirit level) in an oven (not

forced draught) at 50℃ and dried for 4 hours. After 4 hours of drying, the petri dishes were taken from the oven and cooled down to ambient temperature. Their lids were replaced and they were put in a desicator at 25℃ to condition overnight. The desicator contained a saturated calcium nitrate solution, providing a relative humidity (RH) inside the desicator of 50%. After conditioning, the films were taken out of the desicator one by one and were loosened from the petridish they were cast in. Loosening was performed by lifting a section of the film with a small pointed spatula and peeling the film gently from the petridish surface.

3.3.1 Films cast according to the Rotatable Central Composite Design

The Rotatable central composite design of this project will be discussed in section 3.5.1, which will explain the pattern of plasticizer addition in detail. This section explains how the plasticizers were incorporated into the individual films. The plasticizers that were used in film making were glycerol AR (G) (Merck Chemicals (Pty) Ltd, Halfway House, South Africa), polyethylene glycol 400 (PEG) (Merck Chemicals (Pty) Ltd, Halfway House, South Africa) and 90% lactic acid (LA) (BDH Laboratory Supplies, Poole, UK). The plasticizer weights (g) were calculated for each film as a percentage (w/w) of the original weight of the kafirin protein.

$$\frac{\text{Weight of protein (uncorrected)}}{100} \times \%\ \text{plasticizer}$$

According to the Rotatable central composite design, 15 combinations of these three plasticizers were proposed, of which one combination was repeated 6 times. The total therefore, amounted to 20 film sets to be prepared.

Table 2: The different plasticizer combinations according to the Rotatable central composite design, expressed as % (w/w) of the protein

Combination no.	Glycerol (G)	Polyethylene glycol (PEG)	Lactic acid (LA)
1.	3.8	3.8	3.8
2.	12.2	12.2	12.2
3.	12.2	3.8	3.8
4.	3.8	12.2	3.8
5.	3.8	3.8	12.2
6.	12.2	12.2	3.8
7.	12.2	3.8	12.2
8.	3.8	12.2	12.2
9.	1	8	8
10.	15	8	8
11.	8	1	8
12.	8	15	8
13.	8	8	1
14.	8	8	15
*15.	8	8	8

* The repeating plasticizer combination, also served as the standard

The plasticizer combination that was repeated 6 times, served as the standard to which the other films were compared. The plasticizer combinations were randomly divided into 4 groups since the number of films that could be prepared per day was limited. With each group of films made, one film with the repeating plasticizer combination was prepared, serving as the standard.

The film casting method stayed the same as described in section 3.3, except where the total weight of the plasticizers did not amount to 0.6 g. The difference was made up with 70% aqueous ethanol in order to maintain a 13% (w/w) solution of the kafirin in the solvent. The trial with a complete set of twenty films was repeated twice.

3.3.2 Films cast with alternative plasticizers

Films were cast in exactly the same way as described in section 3.3, except for using an alternative plasticizer in combination with LA, instead of G and PEG. In preliminary studies, the ability of LA and SDS (Sodium dodecyl sulphate) (BDH Laboratory Supplies Poole, UK) to dissolve kafirin protein was compared. SDS was found not to dissolve the kafirin protein sufficiently. Therefore, LA was chosen to be used in combination with an alternative plasticizer since the best results were obtained for both protein solvation and plasticization. Glucono-delta-lactone (GDL) (Savannah Fine Chemicals, Johannesburg, South Africa) and a Diacetyl tartaric ester of monoglycerides (DATEM) (Multec Data 2520S, Beldem, South Africa) were each combined with LA in order to obtain a two-component system for film plasticization. The amount of added ethanol was adapted each time in order to maintain a 13% (w/w) kafirin "solution" for each film.

3.3.2.1 Glucono-delta-lactone (GDL)

GDL was applied in different dosages (0 g; 0.1 g; 0.2 g; 0.3 g; 0.4 g; 0.5 g; 0.6 g) in combination with a constant 0.2 g of LA to obtain a dose response curve. The dry GDL powder was weighed out and added directly to the kafirin in the flask. The lactic acid was weighed out separately, mixed with the 70% aqueous ethanol and added to the flask contents. The films were prepared as described in section 3.3. The films where no GDL was present (0 g) served as the control film used to compare the other prepared films to.

3.3.2.2 DATEM

DATEM was also applied in combination with a constant amount of LA (0.2 g). The DATEM was added as a percentage of the protein weight (0%; 1.17%; 2.34%; 3.51%; 4.68%), with the film containing no DATEM serving as the control. The dry DATEM powder was weighed out and added to the kafirin in the flask. The 70% aqueous ethanol and LA were mixed together and added

to the contents of the flask. The films were prepared as discussed in section 3.3.

3.4 Analyses

3.4.1 Protein determination

The protein content of the extracted, defatted kafirin was determined by the Dumas total combustion method, using a Leco FP 528 Protein/Nitrogen Analyzer (St. Joseph, USA). A factor of 6.25 was used for the conversion of total nitrogen to total protein. The protein determination was performed in triplicate.

3.4.2 Film thickness

After conditioning the films overnight in a desicator at 50% RH, they were taken out one at a time, loosened and cut into strips with a scalpel on a cardboard template. From each film, 4 strips (60 mm x 6 mm) and a circle were cut. A circular template with a 40 mm diameter was used to cut the circles. After cutting, the films were not put back in the desicator. A total time of about 5 hours elapsed from cutting the films, measuring the thickness and performing the tensile tests on the film strips. The thickness of the filmstrips and the circle was measured with a micrometer (thickness comparator), graduated to 1 µm. Five thickness measurements were taken over the length of each film, as well as around the edge of the circle.

3.4.3 Sensory evaluation

Before cutting the film, its physical characteristics, clarity, colour, flexibility, surface texture, odour and phase separation were evaluated by the author. A clear, flexible, odourless polyethylene bag with a smooth surface texture was used for comparison. This evaluation was only performed on films made from GDL and DATEM in combination with LA because differences were clearly visible.

3.4.4 Tensile properties

The method used was based on the ASTM D882-97 (American Society for Testing and Materials, 1997) method but modified. A TA-XT2 Texture Analyser (Stable Micro Systems, Goldalming, UK) was used for the texture analysis of the films. The four strips, cut from each film, were mounted one at a time in the grips (Tensile rig grips code A/TG, Stable Micro Systems), 10 mm of each side in the grips, leaving 40 mm exposed. The grips were covered with abrasive paper (Cabinet paper fine, 3 M Construction and Home, Isando, South Africa) to obtain a better grip on the film ends. The settings of the texture analyser were:

Force measured in tension
Pre-test speed: 1.0 mm/s
Test speed: 0.4 mm/s
Post-test speed: 8.0 mm/s
Distance: set according to the expected elongation

Before starting the analysis, the tensile rig was calibrated with a distance set at 40.0 mm. Of the two films from each plasticizer combination, at least 2 strips each were tested. After each test was run, a plot of force (N) on the y-axis against distance (mm) on the x-axis was obtained. The maximum force, force at break and the distance each film stretched were recorded from the computer and the stress and strain were calculated as follows:

$$\text{Stress } (\sigma) \text{ (resistance)} = \frac{\text{F (maximum force)}}{\text{A (width x thickness)}} = \text{N/mm}^2$$

$$\%\ \text{Strain } (\varepsilon) \text{ (extensibility)} = \frac{\text{Increase in length (mm)}}{\text{Original length (mm)}} \times 100$$

3.4.5 Water vapour transmission rate (WVTR) and Water vapour permeability (WVP)

The method used for measuring the water vapour transmission rate (WVTR) and the water vapour permeability (WVP) of the different films was adapted from the ASTM method E96-97 (American Society for Testing and Materials, 1997). For the purpose, Schott bottles (100 ml) were modified by drilling an accurate hole with a 33 mm diameter in the centre of the screw top. In each bottle, 90 ml of water was measured and one of the film circles was placed over the mouth of the bottle. A tap washer (external diameter of 39 mm) was placed on top of the film to cover the rim of the bottle, providing a water-tight seal with the screw top screwed on. Each bottle set-up was weighed individually on a top pan balance to 2 decimal places. The bottles were put on a tray at random and placed in a fume cupboard with the fan switched on for a period of 8 consecutive days. For each of the plasticizer combinations, 2 circles were mounted on bottles at a time and the whole experiment was repeated twice. Each one of the bottles was weighed daily and the weights were recorded. From the recorded weights, a graph of water loss against time was plotted. The best fit of a straight line, with its first point at the origin, was drawn. Out of the line equation, the gradient for each line was calculated. This was used in the calculation of WVTR and WVP:

$$\text{WVTR} = \frac{\text{Gradient of the line (g water lost/h)}}{\text{Area of the film } (m^2)}$$

$$\text{WVP} = \frac{\text{Gradient (g water lost/h) x thickness of the film (mm)}}{\text{Area } (m^2) \times \text{Po (kPa)} \times (\text{RH1-RH2})/100}$$

Where:

Po (at 25℃) = 3.17 kPa.

RH1 = Relative humidity inside the bottle.

RH2 = Relative humidity outside the bottle.

It was assumed that the relative humidity inside the bottle was 100%. A Kane-May KM 8006 Relative humidity meter (Welwyn Garden City, UK) was used to measure the relative humidity outside the bottle.

3.4.6 Dynamic mechanical analysis (DMA)

The films used for the DMA and for the determination of the oxygen permeability (OP) and oxygen transmission rate (OTr), were prepared at the University of Pretoria, South Africa but the analysis itself was performed at the Swedish Institute for Food and Biotechnology, Göteburg, Sweden. The films were prepared as described in section 3.3 and loosened from the petridish. Remaining in its individual petridish, they were put in clearly marked envelopes inside a box and sent to Sweden.

DMA, using a Rheometrics solids analyzer, RSA-II (Rheometric Scientific, Piscataway, USA), was performed under tension at different temperatures on film strips (23 mm), cut from all 15 plasticizer combinations. The method followed was as described by Stading, Rindlav-Westling & Gatenholm (2001). Tests were performed by applying an oscillatory strain to the sample at a constant RH. Each filmstrip was covered with hydrophobic grease before testing to reduce moisture loss. Test frequency was set at 1 Hz, target strain was set to 0.01% (maximum 0.1%) and the temperature range for scans was from -10℃ to 110℃ at 5℃/min.

The following parameters were calculated:
E', called the storage modulus is a measure of the stiffness of the material measured (Anker, 2000). It is proportional to the amount of energy stored in the material.
E", the loss modulus, is proportional to the amount of energy released or given off by the material.
The phase angle, δ, indicates the degree of the materials viscous to elastic behaviour, with δ = 90° indicating a purely viscous material and δ = 0° indicating a purely elastic material. The phase angle is calculated as tan δ = E"/E' (Anker, 2000).

3.4.7 Oxygen permeability (OP) and oxygen transmission rate (OTr)

The oxygen transmission of the films was determined by using the ASTM D 1307-90 method (American Society for Testing and Materials, 1990), also used by Anker, Stading & Hermansson (2000). The analysis was performed on films, containing all 15 plasticizer combinations. A Mocon Oxtran 2/90 (Modern Controls, Minneapolis, USA) was used for the analysis. From each film, a square portion of 25 cm^2 was cut and masked between two aluminum sheets, leaving an opening of 5 cm^2. Before placing the sample in the instrument for analysis, the thickness of the film sample was measured (5 times over the surface). Oxygen permeability, expressed as cm^3 µm m^{-2} $d^{-1}kPa^{-1}$, was read from the instrument. This analysis was performed by the Swedish Institute for Food and Biotechnology on films prepared at the University of Pretoria.

3.4.8 Moisture content

In order to calculate the contribution water made to film plasticization, it was necessary to determine the moisture content of the different plasticizers as well as that of kafirin. The total moisture content (%) per film as contributed by its specific plasticizer combination was calculated as follows:

% Total Moisture = (% plasticizer x % moisture in G) + (% plasticizer x % moisture in PEG) + (% plasticizer x % moisture in LA) + (% moisture in kafirin)

3.4.8.1 Moisture contribution of kafirin

A modified version of the AACC method 44-15A (American Association of Cereal Chemists, 2000) was used to determine the moisture content of films without any plasticizer (only kafirin) in order to be able to calculate how much water the protein contributed to the total moisture content of the films. The analysis was repeated twice. About 1 g of film was weighed out into pre-dried aluminum tins. The total weight was noted and drying was at 103°C for 3

hours. The tins and their contents were weighed again after drying and the % moisture loss was calculated as follows:

$$\% \text{ Moisture} = \frac{\text{Wet weight} - \text{Dry weight}}{\text{Wet weight}} \times 100$$

3.4.8.2 Moisture contribution of different plasticizers

The moisture content of the different plasticizers G, PEG and LA was determined in order to be able to calculate how much water each plasticizer contributes to the total moisture content of each film. The analysis was done in duplicate. Aluminum moisture tins were dried at 103℃ for 1 hour and cooled down in a desicator, containing dry silica crystals. About 5-6 g of each plasticizer was weighed out in the pre-dried moisture tins. The weights of the tins, containing the plasticizers were measured to 4 decimal places. A set of tins containing the different plasticizers was put in a desicator containing a saturated calcium nitrate solution (50% RH) for 48 hours to see how much moisture the different plasticizers absorbed. The moisture content of each plasticizer was calculated as follows:

$$\% \text{ Moisture} = \frac{\text{Weight (before treatment)} - \text{Weight (after treatment)}}{\text{Weight (before treatment)}} \times 100$$

3.4.9 Microscopic analysis of films

3.4.9.1 Light microscopy

In order to obtain thin sections for good light penetration and optimal focus, film samples were embedded in resin. Two film strips of approximately 2 mm x 10 mm in size, were cut from film samples with different plasticizer combinations, as well as from a pure kafirin film with no added plasticizers, serving as the control.

The plasticizer combinations of films prepared and studied by light microscopy were as follows:

1.) 3.8 G; 3.8 PEG; 3.8 LA
2.) 8.0 G; 8.0 PEG; 8.0 LA
3.) 12.2 G; 12.2 PEG; 12.2 LA
4.) 0 G; 0 PEG; 0 LA

The strips were stuck on the inside of the lid of a plastic petri dish using double-sided tape. The strips were then fixed in the vapours of a mixture of 50% glutaraldehyde and 50% formaldehyde for 4 hours. This was followed with a post fixing in osmium tetra oxide vapours for 3 hours. Two sets of strips were prepared, one set was dehydrated in a graded ethanol series and the other set was embedded in resin without dehydration. The dehydration was performed in 50% and 70% ethanol for 10 min each and another 3 times in 100% ethanol, also 10 min each. Both sets of strips were put in a series of Quetol resins (Van der Merwe & Coetzee, 1992) of increasing concentration. In a closed test tube, they were left in a 50% resin solution for 2 hours, followed by a resin concentration of 75% for 12 hours and 100% resin for 4 hours. The films strips were then cut into small pieces (approx. 2 mm x 2 mm) and the pieces were transferred to moulds. Polymerization of the resin took place in an oven at 60℃ for a minimum of 20 hours. Ultra thin sample sections (0.5 µm) were cut with a diamond knife on an ultra-microtome (Reichert Ultracut E, Vienna, Austria), stained with Toluidene blue and dried on a slide warmer. The sections were studied with a transmission light microscope (Nikon Optiphot, Tokyo, Japan) at 40x magnification.

3.5 Statistical design

3.5.1 Rotatable Central Composite Design

For this experiment, a rotatable central composite design approach (Kim *et al.*, 2003) was used to yield an appropriate response surface so that the relationship between three independent variables and a specific film property could be visualized. The three independent variables were the three

plasticizers: G (% (w/w) of the protein weight, X_1), PEG (% (w/w) of the protein weight, X_2) and LA (% (w/w) of the protein weight, X_3). Because these three independent variables were to be tested together with a certain film property, in other words a dependent variable, the results could not be expressed graphically due to the inability to draw in four dimensions. The different film properties under observation were the tensile properties, the glass transition properties, the water vapour barrier properties and the oxygen barrier properties of the films.

The complete design consisted of 20 experimental points in total, of which 6 were the central points of the design. An initial quantity for the three plasticizers was determined in preliminary studies, and the design was constructed around it. The films were prepared in random order to fit time and space limitations. The whole design was repeated twice. All of the statistical calculations were performed with PROC RSREG from SAS® version 8.2 (SAS® System Institute, Carry, NC, USA), running under VM/CMS on a mainframe computer.

The data of each variable was analysed to fit the following second order model:

$$y = \beta_0 - \beta_1 X_1 - \beta_2 X_2 + \beta_3 X_3 - \beta_{11} X_1^2 - \beta_{22} X_2^2 - \beta_{33} X_3^2 - \beta_{12} X_1 X_2 - \beta_{13} X_1 X_3 - \beta_{23} X_2 X_3.$$

Table 3: The Response Surface combinations of the three independent variables in the experimental design

X_1	X_2	X_3
-1	-1	-1
1	-1	-1
-1	1	-1
1	1	-1
-1	-1	1
1	-1	1
-1	1	1
1	1	1
-1.68	0	0
1.68	0	0
0	-1.68	0
0	1.68	0
0	0	-1.68
0	0	1.68
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0
0	0	0

Each of the figures in the table represents a specific amount of each plasticizer used (calculated as a percentage (w/w) of the original weight (g) of kafirin protein):

-1.68 : 1; 1 : 3.8; 0 : 8; 1 : 12.2; 1.68 : 15

3.5.2 Principal component analysis

Principal component analysis was performed on the data obtained from the different analyses that were done on the films. STATISTICA® release 6 (Statsoft, 2001) computer program was used. The matrix of raw data consisted of the three component plasticizer combinations as independent variables and the individual plasticizer amounts as well as the moisture content and different measured film characteristics, such as the tensile properties, the glass transition properties, the water vapour barrier properties and the oxygen permeability as dependent variables.

3.5.3 Analysis of variance (ANOVA)

Analysis of variance, using the least squares procedure was applied to determine whether or not there existed significant differences ($p < 0.05$) between the means of the different measured film characteristics. It was applied to the tensile properties, the glass transition properties as well as the water vapour and oxygen barrier properties of the film.

4. RESULTS

4.1 *The effect of a three-component plasticizer system on kafirin film functional properties*

Table 4: Effect of different plasticizer combinations on the tensile properties of kafirin films

Plasticizers (percentage relative to protein weight) G[a]	PEG[a]	LA[a]	Moisture content (% relative to protein weight)	Thickness mean (µm)	Thickness minimum (µm)	Maximum force (N)	Force at break (N)	Stress[b] (N/mm^2)	Stress[c] (N/mm^2)	Stress at break[b] (N/mm^2)	Stress at break[c] (N/mm^2)	Strain (%)
3.8	3.8	3.8	7.08	102bc[d] (6)[e]	90.0abc (8.0)	8.8abc (5.5)	8.3bcd (5.5)	14.6a (9.2)	16.5ab (10.0)	13.7ab (9.1)	15.5abc (10.0)	1.5a (1.0)
12.2	12.2	12.2	8.79	83a (17)	76.0ab (16.1)	6.2a (3.4)	4.4a (2.4)	12.1a (4.9)	13.3a (5.9)	8.5a (3.7)	9.4a (4.5)	27.8d (19.1)
12.2	3.8	3.8	8.15	89ab (9)	78.8ab (9.9)	12.2cde (2.5)	10.9de (2.0)	23.1cd (4.7)	25.9c (4.1)	20.5cd (3.3)	23.1def (2.8)	4.7a (2.0)
3.8	12.2	3.8	7.71	97abc (15)	83.5abc (14.2)	12.9de (2.9)	12.3e (3.5)	22.1cd (1.8)	25.5c (1.7)	20.9cd (2.9)	24.2ef (2.9)	3.0a (0.2)
3.8	3.8	12.2	7.08	84a (9)	73.0a (8.9)	11.9cde (2.9)	10.4de (2.6)	23.7d (5.2)	27.3c (6.4)	20.6cd (4.3)	23.7def (5.4)	4.3a (1.3)
12.2	12.2	3.8	8.79	98abc (26)	87.5abc (23.2)	13.1de (1.5)	11.9de (1.3)	22.7cd (3.1)	25.6c (3.2)	20.9cd (3.9)	23.5def (3.9)	4.8a (1.1)
12.2	3.8	12.2	8.15	94ab (9)	83.3abc (11.7)	11.7bcde (1.0)	9.9cde (2.0)	20.9cd (0.4)	23.7c (1.7)	17.5bc (2.2)	19.8cde (2.2)	8.2ab (4.8)
3.8	12.2	12.2	7.71	113c (15)	98.8c (4.8)	15.0e (1.0)	13.2e (1.2)	22.6cd (4.0)	25.5c (2.1)	19.8cd (3.1)	22.4def (1.5)	5.2a (2.5)
1.0	8.0	8.0	7.04	90ab (12)	76.8ab (12.3)	12.3cde (3.5)	11.8de (3.8)	22.6cd (3.7)	26.4c (3.3)	21.7cd (4.2)	25.4ef (4.0)	2.7a (0.4)
15.0	8.0	8.0	8.83	94ab (10)	78.3ab (8.5)	11.6bcde (0.3)	9.8cde (0.8)	20.9bcd (1.9)	25.0c (2.1)	17.6bc (1.4)	21.1cdef (1.1)	5.7a (2.3)
8.0	1.0	8.0	7.40	87ab (14)	73.8ab (10.1)	11.5bcde (4.4)	10.7de (4.7)	21.8cd (6.1)	25.5c (6.4)	20.3cd (6.6)	23.7def (7.3)	3.5a (0.7)
8.0	15.0	8.0	8.46	99abc (7)	90.3bc (10.5)	7.8ab (1.7)	5.5ab (1.3)	13.0a (1.9)	14.4a (1.6)	9.2a (1.6)	10.1a (1.7)	18.4c (7.2)
8.0	8.0	1.0	7.93	93ab (5)	80.5ab (4.2)	13.3de (2.8)	13.2e (2.8)	23.8d (4.3)	27.4c (4.7)	23.5d (4.4)	27.1f (4.9)	2.6a (0.3)
8.0	8.0	15.0	7.93	95ab (5)	85.3abc (9.2)	8.4abc (0.4)	6.0abc (0.4)	14.9ab (0.2)	16.7ab (1.0)	10.6a (0.8)	11.9ab (1.6)	15.4bc (7.6)
8.0	8.0	8.0	7.93	96abc (6)	79.0ab (16.0)	10.2bcd (1.9)	8.1abcd (1.7)	17.6abc (2.6)	21.8bc (3.9)	13.9ab (2.1)	17.5bcd (5.0)	8.9ab (6.0)

[a] G = Glycerol, PEG = Polyethylene glycol 400, LA = Lactic acid
[b] Calculated by using the mean film cross sectional area
[c] Calculated by using the minimum film cross sectional area
[d] Values with different letters in the same column are significantly different from each other at the 95% level
[e] Figures in parentheses indicate the standard deviations

In Table 4, the mean film thickness of the films with plasticizer combinations G12.2, PEG12.2, LA12.2 and G3.8, PEG3.8, LA12.2 were the lowest. The film with plasticizer combination G3.8, PEG12.2, LA12.2 was the thickest (about 1.36 times thicker than the thinnest films). In some cases there was a wide variation in thickness within a single treatment e.g. combinations G12.2, PEG12.2, LA3.8 and G12.2, PEG12.2, LA12.2.

Concerning maximum force, plasticizer combination G12.2, PEG12.2, LA12.2 required the least force to deform the film strips. Plasticizer combination G3.8, PEG12.2, LA12.2 gave the highest maximum force value (about 2.4 times the force required to deform film strips from combination G12.2, PEG12.2, LA12.2).

For the force at break, plasticizer combination G12.2, PEG12.2, LA12.2 also gave the lowest value, being most significantly different from plasticizer combinations G3.8, PEG12.2, LA3.8; G3.8, PEG12.2, LA12.2 and G8.0, PEG8.0, LA1.0, which had the highest force at break values (3 times higher than the lowest value).

The values for the stress calculated by using the mean film cross sectional area and the stress calculated by using the minimum film cross sectional area showed similar trends, with the first being the lowest for plasticizer combinations G3.8, PEG3.8, LA3.8; G12.2, PEG12.2, LA12.2 and G8.0, PEG15.0, LA8.0 (about 2 times lower than the largest value). The stress calculated by using the minimum film cross sectional area was also the lowest for combinations G12.2, PEG12.2, LA12.2 and G8.0, PEG15.0, LA8.0. Plasticizer combinations G12.2, PEG12.2, LA12.2; G8.0, PEG15.0, LA8.0 and G8.0, PEG8.0, LA15.0 had the lowest stress at break values, as calculated by using the mean film cross sectional area. Combination G8.0, PEG8.0, LA1.0 had the highest value (about 2.8 times higher than the lowest value). The stress at break, calculated by using the minimum film cross sectional area follows a similar pattern. In general, the same trends occurred for stress and stress at break for the highest and the lowest values of each.

For strain (extensibility), plasticizer combination G12.2, PEG12.2, LA12.2 gave the highest value (18.5 times higher than the lowest value), with combinations G8.0, PEG15.0, LA8.0 and G8.0, PEG8.0, LA15.0 also having values that were quite high (about 10 to 12 times higher than the lowest value). Wide variations in strain within a single treatment did exist, e.g. in combination G12.2, PEG12.2, LA12.2.

Table 5: Effect of different plasticizer combinations on the glass transition properties of kafirin films

Plasticizers (percentage relative to protein weight)			Moisture content (% relative to protein weight)	T_g intersect[b] (°C)	T_g E"peak[c] (°C)	E'($T_g$inters -40°C)[d] (MPa)	δ($T_g$inters -40°C)[e] (°)
G[a]	PEG[a]	LA[a]					
3.8	3.8	3.8	7.08	74.7g[f] (3.1)[g]	71.3j (5.8)	276a (74)	1.17a (0.95)
12.2	12.2	12.2	8.79	42.0abc (0.0)	45.0abc (1.7)	120a (23)	5.20e (0.17)
12.2	3.8	3.8	8.15	60.3e (1.5)	59.7ghi (0.6)	157a (27)	2.70bcd (0.26)
3.8	12.2	3.8	7.71	60.3e (4.0)	56.3fg (2.1)	242a (132)	2.87bcd (1.58)
3.8	3.8	12.2	7.08	66.3f (3.1)	62.7i (2.3)	203a (75)	1.83ab (0.50)
12.2	12.2	3.8	8.79	46.3bc (1.5)	47.0abcd (2.7)	346ab (226)	3.12bcd (0.31)
12.2	3.8	12.2	8.15	47.7c (3.2)	49.0cd (2.7)	167a (64)	3.51cd (0.78)
3.8	12.2	12.2	7.71	54.3d (5.1)	50.3de (1.5)	152a (141)	3.67d (0.49)
1.0	8.0	8.0	7.04	62.0ef (6.2)	61.0hi (3.6)	558b (390)	3.37cd (2.44)
15.0	8.0	8.0	8.83	47.3c (4.5)	48.0bcd (4.4)	255a (82)	3.11bcd (0.30)
8.0	1.0	8.0	7.40	61.7ef (2.5)	57.7fgh (0.6)	153a (57)	3.00bcd (0.57)
8.0	15.0	8.0	8.46	40.7ab (0.6)	43.7ab (1.5)	346ab (25)	2.96bcd (0.47)
8.0	8.0	1.0	7.93	61.0ef (3.0)	57.0fgh (2.7)	171a (73)	2.01abc (0.63)
8.0	8.0	15.0	7.93	40.3a (1.2)	42.7a (1.5)	237a (3)	3.11bcd (0.75)
8.0	8.0	8.0	7.93	57.2de (4.7)	54.5ef (2.8)	233a (51)	2.63abcd (0.54)

[a] G=Glycerol, PEG=Polyethylene glycol 400, LA=Lactic acid
[b] T_g intersect = The glass transition temperature as measured by the temperature where the storage modulus E' of the material tested, changed from glassy state to rubbery state
[c] T_g E"peak = The glass transition temperature as measured by the peak in the loss modulus E"
[d] E'(T_g inters-40°C) = The storage modulus in the glassy state of the material tested
[e] δ(T_g inters-40°C) =The phase angle in the glassy state of the material tested
[f] Values with different letters in the same column are significantly different from each other at the 95% level
[g] Figures in parentheses indicate the standard deviations

In Table 5, T_g intersect temperature was the lowest for plasticizer combination G8.0, PEG8.0, LA15.0 and the highest for plasticizer combination G3.8, PEG3.8, LA3.8 (about 1.85 times higher than the smallest value). Among all the plasticizer combinations, these two combinations were the most significantly different from each other. T_g E"peak showed the same trend as T_g intersect.

No great differences were seen in the data of E' (T_g inters-40℃), obtained for the different plasticizer combinations. The value for plasticizer combination G1.0, PEG8.0, LA8.0 was significantly different from most of the others, except for G12.2, PEG12.2, LA3.8 and G8.0, PEG15.0, LA8.0.

For δ (T_g inters-40℃), the phase angle in the glassy state of the material, the greatest significant difference was between plasticizer combinations G3.8, PEG3.8, LA3.8 and G12.2, PEG12.2, LA12.2. Combination G3.8, PEG3.8, LA3.8 had the smallest value and G12.2, PEG12.2, LA12.2 had the largest value (4.4 times bigger than the smallest value).

Table 6: Effect of different plasticizer combinations on the moisture barrier properties of kafirin films

Plasticizers (percentage relative to protein weight)			Moisture content (% relative to protein weight)	Thickness mean (µm)	WVTR[b] (g/h/m^2)	WVP[c] (gmm/m^{2}hkPa)
G[a]	PEG[a]	LA[a]				
3.8	3.8	3.8	7.08	98bcd[d] (6)[e]	5.91a (0.22)	0.242a (0.006)
12.2	12.2	12.2	8.79	89abcd (25)	10.17e (2.60)	0.360ef (0.067)
12.2	3.8	3.8	8.15	82abc (18)	8.12bcd (0.41)	0.278abcd (0.049)
3.8	12.2	3.8	7.71	101cd (6)	7.09ab (0.18)	0.302bcd (0.012)
3.8	3.8	12.2	7.08	89abcd (18)	6.78ab (0.87)	0.251ab (0.040)
12.2	12.2	3.8	8.79	92abcd (17)	8.61cd (0.26)	0.333def (0.053)
12.2	3.8	12.2	8.15	85abc (11)	7.84bc (0.30)	0.278abcd (0.027)
3.8	12.2	12.2	7.71	107d (16)	6.90ab (0.31)	0.308cdef (0.033)
1.0	8.0	8.0	7.04	90abcd (11)	6.13a (0.26)	0.230a (0.018)
15.0	8.0	8.0	8.83	81ab (12)	8.92cde (0.34)	0.303bcd (0.032)
8.0	1.0	8.0	7.40	75a (11)	7.15ab (0.12)	0.225a (0.034)
8.0	15.0	8.0	8.46	85abc (14)	9.48de (2.01)	0.333def (0.051)
8.0	8.0	1.0	7.93	95bcd (8)	7.65bc (0.54)	0.305bcde (0.022)
8.0	8.0	15.0	7.93	99bcd (8)	8.77cde (1.38)	0.362f (0.050)
8.0	8.0	8.0	7.93	81ab (12)	8.08bcd (0.31)	0.274abc (0.043)

[a] G=Glycerol, PEG=Polyethylene glycol 400, LA=Lactic acid
[b] WVTR = Water vapour transmission rate
[c] WVP = Water vapour permeability
[d] Values with different letters in the same column are significantly different from each other at the 95% level
[e] Figures in parentheses indicate the standard deviations

In Table 6, the thickness of the film with plasticizer combination G8.0, PEG1.0, LA8.0 was the lowest. Plasticizer combination G3.8, PEG12.2, LA12.2 gave the thickest film (1.43 times thicker than the thinnest films). Wide variations in the film thickness within a single treatment occurred, e.g. plasticizer combinations G12.2, PEG12.2, LA12.2; G12.2, PEG3.8, LA 3.8 and G12.2, PEG12.2, LA 3.8.

The WVTR of the films with plasticizer combinations G3.8, PEG3.8, LA3.8 and G1.0, PEG8.0, LA8.0 were statistically the same and the lowest. Plasticizer combination G12.2, PEG12.2, LA12.2 gave the highest WVTR. The WVTR of plasticizer combination G12.2, PEG12.2, LA12.2 were 1.72 times higher than that of plasticizer combination G3.8, PEG3.8, LA3.8 with the lowest WVTR.

The same trend could be seen with WVP. Plasticizer combinations G3.8, PEG3.8, LA3.8; G1.0, PEG8.0, LA8.0 and G8.0, PEG1.0, LA8.0 gave the lowest WVP. The highest WVP was for plasticizer combinations G12.2, PEG12.2, LA12.2 and G8.0, PEG8.0, LA15.0 (about 1.6 times higher than the lowest WVP).

Table 7: Effect of different plasticizer combinations on the oxygen permeability of kafirin films

Plasticizers (percentage relative to protein weight)			Moisture content (% relative to protein weight)	Thickness Mean (µm)	O Tr[b] (cm^3/m^2d)	OP[c] ($cm^3µm/m^2.d.kPa$)
G[a]	PEG[a]	LA[a]				
3.8	3.8	3.8	7.08	105bc[d] (0)[e]	53.5abc (3.5)	56cd (4)
12.2	12.2	12.2	8.79	105bc (21)	84.5ef (7.8)	87f (10)
12.2	3.8	3.8	8.15	84a (5)	61.5cd (5.0)	51bc (1)
3.8	12.2	3.8	7.71	97abc (1)	61.5cd (2.1)	59de (1)
3.8	3.8	12.2	7.08	91ab (6)	48.5ab (0.7)	44ab (2)
12.2	12.2	3.8	8.79	94abc (9)	87.0f (6.7)	80f (1)
12.2	3.8	12.2	8.15	97abc (16)	59.7bc (12.1)	56cd (1)
3.8	12.2	12.2	7.71	94abc (9)	87.0f (6.7)	80f (1)
1.0	8.0	8.0	7.04	94abc (11)	55.4abc (6.7)	51c (0)
15.0	8.0	8.0	8.83	112c (3)	72.5de (3.9)	80f (3)
8.0	1.0	8.0	7.40	98abc (13)	45.3a (6.1)	43a (0)
8.0	15.0	8.0	8.46	106bc (11)	97.2f (8.8)	101g (1)
8.0	8.0	1.0	7.93	95abc (6)	53.4abc (3.3)	50abc (0)
8.0	8.0	15.0	7.93	96abc (6)	62.5cd (2.1)	59de (7)
8.0	8.0	8.0	7.93	103abc (4)	65.1cd (2.5)	66e (0)

[a] G=Glycerol, PEG=Polyethylene Glycol 400, LA=Lactic acid
[b] OTr = Oxygen transmission rate
[c] OP = Oxygen permeability
[d] Values with different letters in the same column are significantly different from each other at the 95% level
[e] Figures in parentheses indicate the standard deviation

In Table 7, the film with plasticizer combination G12.2, PEG3.8, LA3.8 was the thinnest. The thickest film was the one made with plasticizer combination G15.0, PEG8.0, LA8.0 and had a thickness about 1.3 times bigger than that of the thinnest film. Wide variations in film thickness within a single treatment occurred, e.g. plasticizer combinations G12.2, PEG12.2, LA12.2 and G12.2, PEG3.8, LA12.2.

Plasticizer combination G8.0, PEG1.0, LA8.0 gave the lowest OTr (2.1 times less than the highest OTr). Plasticizer combinations G12.2, PEG12.2, LA3.8; G3.8, PEG12.2, LA12.2 and G8, PEG15.0, LA8.0 were statistically the same, all giving the highest OTr.

Plasticizer combination G8.0, PEG1.0, LA8.0 also gave the lowest OP (2 times lower than the highest OP). Plasticizer combinations G12.2, PEG12.2, LA12.2; G12.2, PEG12.2, LA3.8; G3.8, PEG12.2, LA12.2 and G15.0, PEG8.0, LA8.0 were all statistically the same and gave the highest OP.

Table 8: Statistical analysis performed with PROC RSREG of the tensile properties of the kafirin films; estimated regression coefficients

Coefficient	Dependant variables					
	Maximum force	**P-value**	**Force at Break**	**P-value**	**Strain**	**P-value**
β_0	5.012	0.201	12.418	0.017	49.000	0.051
β_1	-0.254	0.385	-0.660	0.073	-4.139	0.035
β_2	0.175	0.544	-0.186	0.579	-2.124	0.235
β_3	-0.698	0.033	-1.222	0.004	-2.336	0.195
β_{11}	-0.008	0.513	0.006	0.683	0.172	0.047
β_{22}	-0.021	0.129	-0.014	0.370	0.047	0.548
β_{33}	0.017	0.200	0.033	0.052	0.019	0.811
β_{12}	-0.019	0.322	-0.007	0.743	0.163	0.171
β_{13}	0.021	0.260	0.027	0.215	0.244	0.045
β_{23}	0.002	0.899	0.014	0.485	0.160	0.142
[a] Mthick	117.679	0.004	79.183	0.056	-266.453	0.185
[b] R-Square	0.937		0.937		0.905	

Model for analysis of property values, using X_1=G (% (w/w) of the kafirin protein weight), X_2=PEG (% (w/w) of the kafirin protein weight) and X_3=LA (% (w/w) of the kafirin protein weight): $Y = \beta_0 - \beta_1X_1 - \beta_2X_2 + \beta_3X_3 - \beta_{11}X_1^2 - \beta_{22}X_2^2 - \beta_{33}X_3^2 - \beta_{12}X_1X_2 - \beta_{13}X_1X_3 - \beta_{23}X_2X_3 + \beta_n\text{Mthick} + \varepsilon$

ε = Residual for the Y measurement

[a] Mthick = Film thickness, a covariable

[b] R-Square = Goodness of fit measurement

In Table 8, a summary is given of the regression coefficients of the tensile data of the films with different plasticizer combinations. The covariable, film thickness that was taken into account had an influence on the maximum force ($P \leq 0.05$), whereas its influence on force at break and strain (extensibility) was not significant. The high R-square values for maximum force, force at break and strain were close to 1, indicating that the data were adequately explained, i.e. that the model fitted the data quite well. The stationary point for each of the three dependent variables was found to be a saddle point. This means that no maximum or minimum value for the different variables could be found.

Table 9: Statistical analysis performed with PROC RSREG of the glass transition (T_g) properties of the kafirin films; estimated regression coefficients

	Dependant variables							
Coefficient	**T_g inter[a]**	**P-value**	**T_gE" peak[b]**	**P-value**	**E'(T_g-inter)[c]**	**P-value**	**δ(T_g-inter)[d]**	**P-value**
B_0	105.744	<0.0001	86.223	<0.0001	401.704	0.173	4.697	0.017
B_1	-3.258	0.050	-2.057	0.107	-81.187	0.033	-0.153	0.456
B_2	-2.868	0.078	-1.616	0.194	7.695	0.819	-0.280	0.187
B_3	-1.729	0.264	-0.599	0.617	27.049	0.428	-0.280	0.186
B_{11}	0.037	0.580	0.015	0.783	3.584	0.035	0.004	0.632
B_{22}	-0.034	0.615	-0.057	0.301	-0.109	0.942	0.011	0.222
B_{33}	-0.054	0.425	-0.067	0.227	-1.038	0.495	0.002	0.798
B_{12}	0.099	0.279	0.078	0.284	1.892	0.353	0.000	1.000
B_{13}	0.028	0.750	0.007	0.920	-0.092	0.963	0.020	0.121
B_{23}	0.127	0.172	0.078	0.284	-1.509	0.455	0.020	0.121
[e] R-Square	0.905		0.894		0.564		0.646	

Model for analysis of property values, using X_1=G (% (w/w) of the kafirin protein weight), X_2=PEG (% (w/w) of the kafirin protein weight) and X_3=LA (% (w/w) of the kafirin protein weight): $Y = \beta_0 - \beta_1X_1 - \beta_2X_2 + \beta_3X_3 - \beta_{11}X_1^2 - \beta_{22}X_2^2 - \beta_{33}X_3^2 - \beta_{12}X_1X_2 - \beta_{13}X_1X_3 - \beta_{23}X_2X_3 + \varepsilon$

ε = Residual for the Y measurement

[a] T_g intersect = The glass transition temperature as measured by the temperature where the storage modulus E' of the material tested, changed from glassy state to rubbery state

[b] T_g E" peak = The glass transition temperature as measured by the peak in the loss modulus E"

[c] E' (T_g intersect) = The storage modulus in the glassy state of the material tested

[d] δ (T_g intersect) = The phase angle in the glassy state of the material tested

[e] R-Square = Goodness of fit measurement

In Table 9, for the glass transition properties it was not necessary to take into account any covariable, since no additional factors were present that could influence the data. The R-square values for the T_g intersect and the T_gE" peak were very close to 1, indicating that the data have been explained well. The R-square values for E'(T_g intersect) and δ(T_g intersect) were low. This indicates that the model did not fit the data very well, explaining only part of the data. The stationary points for each of the four dependent variables were again found to be saddle points.

Table 10: Statistical analysis performed with PROC RSREG of the water barrier properties of the kafirin films; estimated regression coefficients

Coefficient	Dependent variables					
	WVTR[a]	P-value	WVP[b]	P-value	Gradient[c]	P-value
β_0	-0.114×10^{-3}	0.001	-2.011×10^{-5}	0.002	-5.829×10^{-2}	0.027
β_1	-4.644×10^{-6}	0.018	-0.809×10^{-6}	0.201	-6.827×10^{-3}	0.030
β_2	-4.007×10^{-6}	0.033	-0.680×10^{-6}	0.277	-5.405×10^{-3}	0.073
β_3	-0.489×10^{-6}	0.816	1.178×10^{-6}	0.074	-5.564×10^{-3}	0.066
β_{11}	6.797×10^{-8}	0.365	2.546×10^{-8}	0.359	5.967×10^{-5}	0.633
β_{22}	8.229×10^{-8}	0.277	1.166×10^{-8}	0.669	0.110×10^{-3}	0.383
β_{33}	-9.481×10^{-8}	0.272	-6.066×10^{-8}	0.045	3.361×10^{-5}	0.787
β_{12}	5.151×10^{-8}	0.604	3.130×10^{-8}	0.393	-6.996×10^{-6}	0.966
β_{13}	8.983×10^{-8}	0.422	-3.033×10^{-8}	0.407	0.308×10^{-3}	0.083
β_{23}	0.148×10^{-6}	0.178	-7.301×10^{-9}	0.839	0.331×10^{-3}	0.066
[d] Mthick	0.530×10^{-3}	0.012	-	-	-	-
[e] R-Square	0.929		0.740		0.853	

Model for analysis of property values, using X_1=G (% (w/w) of the kafirin protein weight), X_2=PEG (% (w/w) of the kafirin protein weight) and X_3=LA (% (w/w) of the kafirin protein weight): $Y = \beta_0 - \beta_1X_1 - \beta_2X_2 + \beta_3X_3 - \beta_{11}X_1^2 - \beta_{22}X_2^2 - \beta_{33}X_3^2 - \beta_{12}X_1X_2 - \beta_{13}X_1X_3 - \beta_{23}X_2X_3 + \varepsilon$

ε = Residual for the Y measurement

[a] WVTR = Water vapour transmission rate

[b] WVP = Water vapour permeability

[c] Gradient = Slope of the regression line of the graph of water loss against time, calculated for the best fitting straight line drawn through the origin of the graph

[d] Mthick = film thickness, a covariable; only applicable to WVT since film thickness is already taken into account in the WVP calculation

[e] R-Square = Goodness of fit measurement

In Table 10, the covariable film thickness was taken into account for WVTR and it can be seen that film thickness definitely had an influence on the water vapour transmission properties of kafirin films ($P \leq 0.05$). Thickness was already included in the calculation of WVP. The R-square value of WVTR is the closest to 1, indicating that the data were adequately explained, with the R-square values of the gradient and WVP in decreasing order. Their R-square values are still close to 1, meaning that there is still a good fit between the model and the data. The stationary point for each of the three dependent variables was again found to be saddle points.

Table 11: Statistical analysis performed with PROC RSREG of the Oxygen barrier properties of the kafirin films; estimated regression coefficients

Coefficient	Dependant variables			
	OTr[a]	P-value	OP[b]	P-value
β_0	30.213	0.079	52.377	0.293
β_1	2.591	0.192	-4.363	0.458
β_2	-0.961	0.615	5.867	0.323
β_3	2.117	0.279	-2.396	0.680
β_{11}	0.003	0.970	0.287	0.282
β_{22}	0.146	0.108	-0.509	0.072
β_{33}	-0.099	0.260	0.063	0.809
β_{12}	0.021	0.850	0.142	0.681
β_{13}	-0.191	0.112	0.014	0.967
β_{23}	0.205	0.090	0.241	0.488
[c] R-Square	0.916		0.484	

Model for analysis of property values, using X_1=G (% (w/w) of the kafirin protein weight), X_2=PEG (% (w/w) of the kafirin protein weight) and X_3=LA (% (w/w) of the kafirin protein weight): $Y = \beta_0 - \beta_1X_1 - \beta_2X_2 + \beta_3X_3 - \beta_{11}X_1^2 - \beta_{22}X_2^2 - \beta_{33}X_3^2 - \beta_{12}X_1X_2 - \beta_{13}X_1X_3 - \beta_{23}X_2X_3 + \varepsilon$
ε = Residual for the Y measurement
[a] OTr = Oxygen transmission rate
[b] OP = Oxygen permeability
[c] R-Square = Goodness of fit measurement

In Table 11, for the OTr and the OP data, again no covariable was taken into account. The R-square value of OTr was very close to 1, indicating a good explanation of the data, but the low R-square value of OP indicates that the model did not fit the data very well, explaining only part of it. The stationary points for the two dependent variables were again saddle points.

Table 12: Summary of the estimated responses for the different film properties as influenced by the three plasticizers, as determined with PROC RSREG

Property	G[a]	PEG[a]	LA[a]	Response
Maximum Force	↓↓	↓	↓↓	↑
Force at Break	↓	↓	↓↓	↑↑
Strain	↑↑	↑	↑	↑↑↑↑
[b] T_g intersect	↓↓	↓↓	↓	↑
[c] Tg E"peak	↓↓	↓↓	↓	↑
[d] E'(T_g intersect)	↓↓	→	→	↑↑↑
[e] δ(T_g intersect)	↑	↑↑	↑↑	↑↑
[f] WVTR	↓↓	↓	↓	↓
[g] WVP	↓↓	↓↓	→	↓
[h] Gradient	↓↓	↓	↓	↓
[i] OTr	↑	↑↑	↑	↑
[j] OP	↑↑	↑	↑	↑

↑ 0-50% increase; ↑↑ 50-100% increase; ↑↑↑ 100-200% increase; ↑↑↑↑ >200% increase
↓ 0-50% decrease; ↓↓ 50-100% decrease; ↓↓↓ 100-200% decrease; ↓↓↓↓ >200% decrease
→ No change
[a] G = Glycerol, PEG = Polyethylene glycol, LA = Lactic acid
[b] T_g intersect = The glass transition temperature as measured by the temperature where the storage modulus E' of the material tested, changed from glassy state to rubbery state
[c] T_g E"peak = The glass transition temperature as measured by the peak in the loss modulus E"
[d] E' (T_g intersect) = The storage modulus in the glassy state of the material tested
[e] δ(T_g intersect) = The phase angle in the glassy state of the material tested
[f] WVTR = Water vapour transmission rate
[g] WVP = Water vapour permeability
[h] Gradient = Slope of the regression line of the graph of water loss against time, calculated for the best fitting straight line drawn through the origin of the graph
[i] OTr = Oxygen transmission rate
[j] OP = Oxygen permeability

Table 12 shows the changes in film properties that would result from an increase or a decrease in each of the three plasticizers. It can clearly be seen that none of the plasticizers can be isolated as having an individual effect on any of the film properties. It seems as if the plasticizers work synergistically to influence the film properties. The only case where an effect took place in isolation was where E'(T_g intersect) was influenced by a lowering in G, without any change in PEG or LA. The WVP of the films was also affected only by G and PEG, without LA playing a role.

It is estimated that film strain (extensibility) will increase radically (>200%) if the amount of all three plasticizers added is increased. In this case, G needs to increase more than PEG or LA. Maximum force and force at break will increase if all three plasticizers decrease.

A great increase (100-200%) in E'(T_g intersect) will take place if G decreases. PEG and LA do not seem to be very influential. T_g intersect and T_g E"peak will increase if all three plasticizers decrease, but δ(T_g intersect) will increase only if all three plasticizers increase.

WVTR, WVP and the gradient all seem to follow the same trend of decreasing if G, PEG and LA decrease. For WVP though, PEG needs to decrease more substantially than for WVTR and for the gradient, and LA should stay constant.

OTr and OP will increase with a corresponding increase in all three plasticizers. A greater increase in G is required for an increase in OP and a greater increase in PEG for OTr.

Table 13: Correlations between the different film characteristics, including the different plasticizers and the three principal components

Variable	Correlations		
	PC[a] 1	PC[a] 2	PC[a] 3
Percentage of variance accounted for	56.06	12.65	12.05
G[b]	-0.574*	0.574*	-0.433
PEG[b]	-0.680**	-0. 001	0.665**
LA[b]	-0.382	-0.502	-0.418
Moisture (%)	-0.840***	0.493	-0.034
Stress (N/mm^2)[c]	0.598*	0.653**	0.083
Stress at Break (N/mm^2)[d]	0.686**	0.616*	0.168
Strain (%)	-0.860***	-0.364	-0.213
T_g intersect (°C)[e]	0.932***	-0.091	0.048
T_g E" peak (°C)[f]	0.907***	-0.147	0.033
E'(T_g intersect) (MPa)[g]	0.136	-0.224	0.722**
δ(T_g intersect) (°)[h]	-0.704**	0.017	-0.105
WVTR ($g/h/m^2$)[i]	-0.919***	0.183	-0.190
WVP (gmm/m^2hkPa)[j]	-0.858***	0.125	0.067
OTr (cm^3/m^2d)[k]	-0.829***	0.067	0.434
OP ($cm^3\mu m/m^2.d.kPa$)[l]	-0.850***	-0.020	0.397

*Significant at 5% level, **Significant at 1% level, ***Significant at 0.1% level
[a] PC = Principal component
[b] G=Glycerol, PEG=Polyethylene glycol 400, LA=Lactic acid
[c] Stress, calculated by using the mean film cross sectional area
[d] Stress at break, calculated by using the mean film cross sectional area
[e] T_g intersect = The glass transition temperature as measured by the temperature where the storage modulus E' of the material tested, changed from glassy state to rubbery state
[f] T_g E"peak = The glass transition temperature as measured by the peak in the loss modulus E"
[g] E' (T_g intersect) = The storage modulus in the glassy state of the material tested
[h] δ(T_g intersect) = The phase angle in the glassy state of the material tested
[i] WVTR = Water vapour transmission rate
[j] WVP = Water vapour permeability
[k] OTr = Oxygen transmission rate
[l] OP = Oxygen permeability

a.

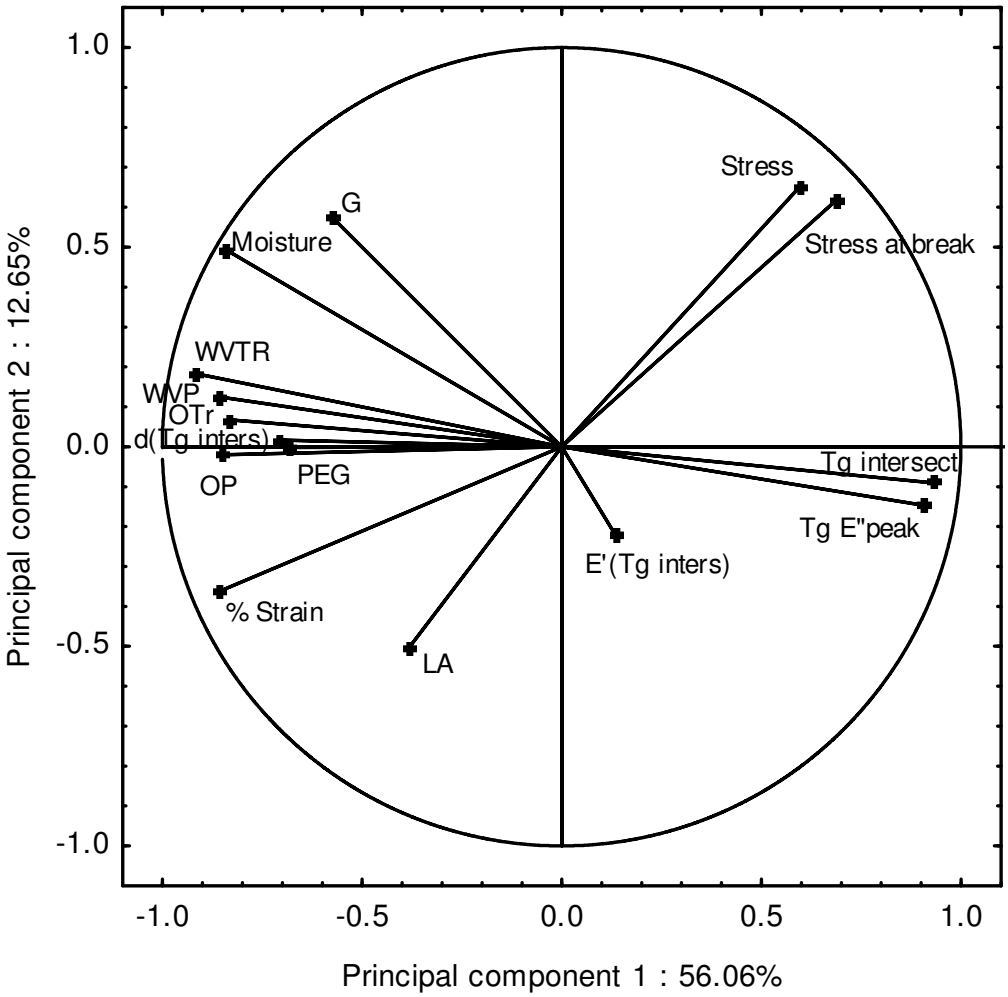
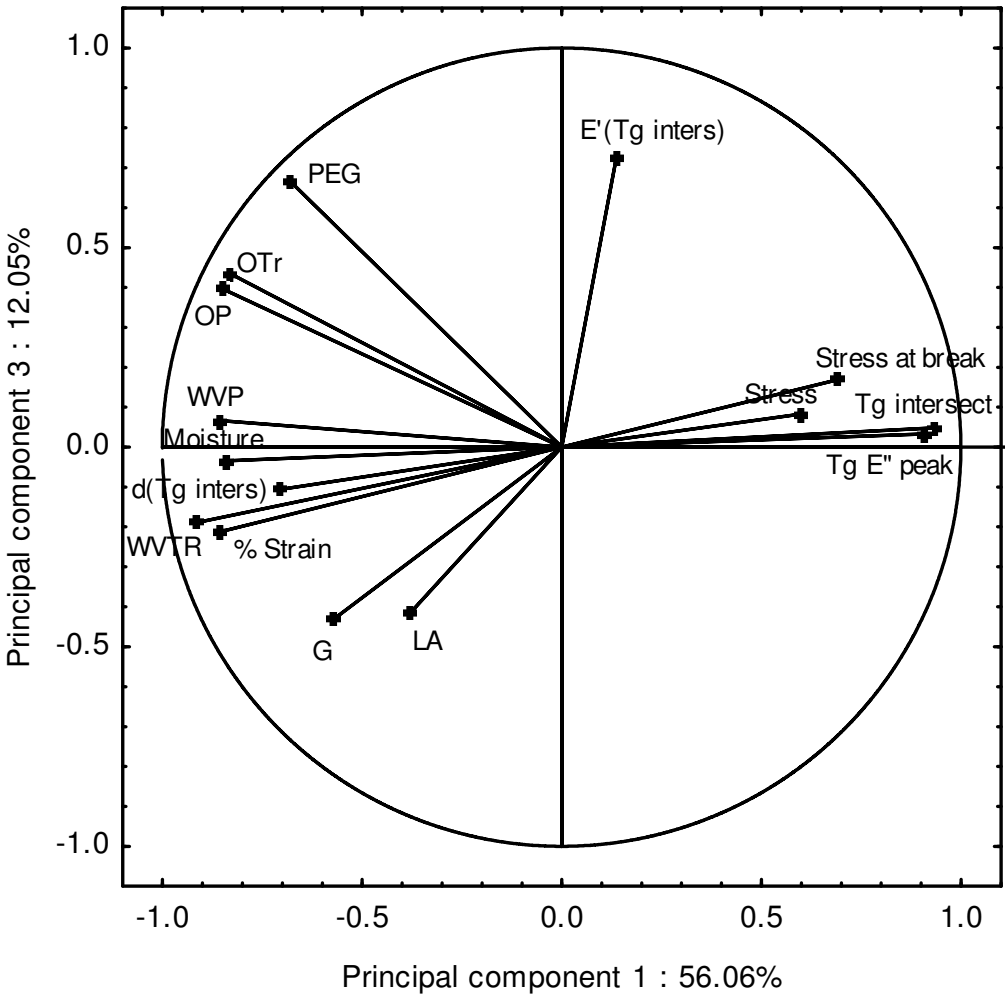


Fig 11: Principal component analysis to show the effect of plasticization on different film characteristics, illustrated on three principal components

a. Principal components 1 x 2

b.



G = Glycerol, PEG = Polyethylene glycol 400, LA = Lactic acid
Stress, calculated by using the mean film cross sectional area
Stress at break, calculated by using the mean film cross sectional area
T_g intersect = The glass transition temperature as measured by the temperature where the storage modulus E' of the material tested, changed from glassy state to rubbery state
T_g E"peak = The glass transition temperature as measured by the peak in the loss modulus E"
E' (T_g intersect) = The storage modulus in the glassy state of the material tested
δ(T_g intersect) = The phase angle in the glassy state of the material tested
WVTR = Water vapour transmission rate
WVP = Water vapour permeability
OTr = Oxygen transmission rate
OP = Oxygen permeability

b. Principal components 1 x 3

From Table 13 and Figure 11, it can be seen that principal component 1 described 56.06% of the data, i.e. carrying the most weight of the three factors. Principal component 2 described 12.65% and principal component 3 describes an almost equal 12.05% of the data. In total, all three principal components accounted for 80.76% of the data, which is a very good interpretation of the data. In principal component 1, G and PEG were both significant, PEG more than G. LA was not significant in factor 1. The moisture content of the films was very significant in factor 1 and positively correlated to G and PEG. In other words, if the plasticizer content of the films increases, the moisture content will also increase. Stress and stress at break were both significant in principal component 1. Stress at break was more significant than stress, but both were negatively correlated with G, PEG and moisture content. Strain was very significant in principal component 1, being positively correlated with plasticizers G and PEG, as well as to moisture content. The barrier properties of the films, both water vapour and oxygen were strongly represented in principal component 1. WVP, WVTR, OP and OTr were all positively correlated with plasticizers G and PEG, as well as to moisture.

Among the glass transition temperature parameters, T_g intersect and T_g E" peak were very strongly negatively correlated with plasticizers G and PEG, and very negatively correlated with the barrier properties of the films. A very strong negative correlation with moisture content also existed. The exception was $\delta(T_g$ inters), which was negatively correlated to the above-mentioned T_g properties but positively correlated with the barrier properties and plasticizers. All of these film characteristics were mostly represented in principal component 1.

Because principal components 2 and 3 described so little of the data, there were only a few of the variables that were significant in those two planes. LA was not found to be significant in any of the principal components. It was interesting to see that G was positively correlated with stress and stress at break in principal component 2. In principal component 3, PEG was the only plasticizer that was significantly represented, being positively correlated with E'(T_g inters-40℃).

4.2 The effect of a two-component plasticizer system on kafirin film functional properties

Table 14: Effect of different DATEM concentrations on the tensile properties of kafirin films

Plasticizer (percentage relative to protein weight) DATEM[a]	Thickness mean (µm)	Thickness minimum (µm)	Maximum force (N)	Force at break (N)	Stress[b] (N/mm^2)	Stress[c] (N/mm^2)	Stress at break[b] (N/mm^2)	Stress at break[c] (N/mm^2)	Strain (%)
0	77.8a[d] (9.9)[e]	66.8a (13.5)	11.0a (2.4)	9.6a (2.6)	23.2ab (2.4)	27.3b (1.1)	20.2a (3.9)	23.5ab (2.7)	3.68b (1.64)
1.17	80.8a (13.2)	70.3a (14.4)	12.3a (2.8)	11.2a (2.9)	25.1b (2.7)	29.0b (1.7)	22.9a (2.6)	26.4bc (1.9)	2.90ab (0.81)
2.34	90.5a (13.0)	77.5a (22.2)	10.5a (2.7)	10.3a (2.7)	19.2a (2.4)	22.9a (1.1)	18.7a (2.6)	22.3a (1.1)	2.27ab (0.57)
3.51	82.0a (13.4)	69.5a (13.7)	11.5a (3.0)	11.3a (2.9)	23.1ab (3.5)	27.4b (2.2)	22.8a (3.3)	27.0c (2.0)	2.18a (0.49)
4.68	81.0a (16.4)	71.0a (23.5)	10.6a (4.2)	10.2a (4.0)	21.0ab (4.3)	24.4a (2.1)	20.4a (4.1)	23.7ab (1.8)	2.51ab (0.85)

A constant amount (0.2 g) of LA was added together with the DATEM to each film
[a] DATEM = Diacetyl tartaric ester of monoglyceride
[b] Calculated by using the mean film cross sectional area
[c] Calculated by using the minimum film cross sectional area
[d] Values with different letters in the same column are significantly different from each other at the 95% level
[e] Figures in parentheses indicate the standard deviation

In Table 14, there was no significant difference between the values for mean film thickness or minimum film thickness, therefore it did not seem as if DATEM had an effect on thickness. Wide variations in film thickness within a single treatment did occur though, e.g. the film with 4.68 % DATEM. The values for maximum force and force at break did also not differ from each other, irrespective of the DATEM concentration.

The values for stress were very similar, even though statistically some did differ. The values of the stress calculated by using the mean film cross sectional area for the films with 1.17% added DATEM and 2.34% added DATEM differed from each other significantly. The value for the film with 2.34% DATEM was the lowest and for the 1.17% film the highest. For stress, calculated by using the minimum film cross sectional area there was the same trend. The lowest values, those of the films with 2.34% and 4.68% added DATEM did not differ significantly from each other. The values of the films with 0%, 1.17% and 3.51% added DATEM also did not differ significantly from each other. They had the highest values for stress, calculated with the minimum film cross sectional area.

For stress at break, calculated by using the mean film cross sectional area there was no significant difference between the treatments. For stress at break, calculated by using the minimum film cross sectional area, the film with 2.34% added DATEM had the lowest value and the film with 3.51% DATEM had the highest value.

The values for the strain (extensibility) were different, however. The film with no added DATEM had the highest extensibility and the film with 3.51% DATEM had the lowest extensibility. This is not logical.

Table 15: Effect of different DATEM concentrations on the moisture barrier properties of kafirin films

Plasticizer (percentage relative to protein weight) DATEM[a]	Thickness mean (µm)	WVTR ($g/h/m^2$)[b]	WVP (gmm/m^2hkPa)[c]
0	80a[d]	5.38a	0.182ab
	(13)[e]	(0.28)	(0.034)
1.17	74a	5.60a	0.173a
	(12)	(0.39)	(0.029)
2.34	102b	5.22a	0.223b
	(19)	(0.27)	(0.041)
3.51	85ab	5.22a	0.187ab
	(16)	(0.42)	(0.033)
4.68	91ab	5.22a	0.198ab
	(8)	(0.61)	(0.014)

A constant amount (0.2 g) of LA was added together with the DATEM to each film
[a] DATEM = Diacetyl tartaric ester of monoglyceride
[b] WVTR = Water vapour transmission rate
[c] WVP = Water vapour permeability
[d] Values with different letters in the same column are significantly different from each other at the 95% level
[e] Figures in parentheses indicate the standard deviation

In Table 15, the thicknesses of the films with 0% and 1.17% added DATEM did not differ from each other. They were the thinnest of all the films, and the film containing 2.34% DATEM was the thickest. Wide variations in the film thickness within a single treatment were present, e.g. the films with 2.34% DATEM and 3.51% DATEM. There was no significant difference between the different treatments in terms of WVTR. A significant difference was seen though between the WVP values of the different films. The film with 1.17% added DATEM had the lowest WVP and the film with 2.34% added DATEM had the highest WVP.

Sensory evaluation of films with added DATEM:

The films with added DATEM and the control with no DATEM were sensorically evaluated by comparison with a clear polythene bag. All of the kafirin films scored quite high on clarity, all had a similar yellow colour, a mild odour and a relatively smooth surface texture. Generally, the films with more DATEM appeared to be a little more flexible when compared to the control, but this was not very obvious.

Table 16: Effect of different GDL concentrations on the tensile properties of kafirin films

Plasticizer (percentage relative to protein weight) GDL[a]	**Thickness mean (µm)**	**Thickness minimum (µm)**	**Maximum force (N)**	**Force at break (N)**	**Stress[b] (N/mm^2)**	**Stress[c] (N/mm^2)**	**Stress at break[b] (N/mm^2)**	**Stress at break[c] (N/mm^2)**	**Strain (%)**
0	84a[d] (11)[e]	77.8a (7.4)	14.2e (2.3)	13.8d (2.7)	28.6f (4.8)	30.7d (4.9)	27.5e (4.7)	29.6d (5.1)	2.6a (1.4)
6.94	102b (5)	95.5b (8.7)	14.2e (0.6)	13.8d (0.9)	23.2e (0.6)	25.0c (1.8)	22.6d (1.2)	24.4c (2.3)	2.8a (0.8)
13.89	99b (6)	86.5ab (2.4)	11.4d (1.8)	10.7c (2.3)	19.3d (2.6)	22.0c (3.6)	18.0c (3.4)	20.6c (4.5)	5.6a (2.1)
20.83	101b (10)	93.8b (15.3)	9.2c (0.7)	7.5b (1.3)	15.4c (2.4)	17.0b (4.4)	12.5b (3.2)	14.0b (5.1)	13.8ab (6.5)
27.78	105b (2)	96.5b (7.0)	8.3bc (1.1)	6.6ab (1.0)	13.4bc (1.6)	14.5ab (1.3)	10.6ab (1.4)	11.5ab (1.0)	13.0ab (4.4)
34.72	106b (4)	99.5b (3.8)	7.0ab (0.4)	5.6ab (0.8)	11.0ab (1.0)	11.7a (1.2)	8.9ab (1.6)	9.5ab (1.8)	21.0b (20.1)
41.67	105b (14)	91.5ab (16.3)	6.0a (0.6)	4.8a (0.7)	9.7a (0.9)	11.2a (1.6)	7.7a (1.0)	8.9a (1.3)	23.1b (13.0)

A constant amount (0.2 g) of LA was added together with the GDL to each film
[a] GDL = Glucono-delta-Lactone
[b] Calculated by using the mean film cross sectional area
[c] Calculated by using the minimum film cross sectional area
[d] Values with different letters in the same column are significantly different from each other at the 95% level
[e] Figures in parentheses indicate the standard deviation

In Table 16, the films with added GDL all generally had the same thickness. The control film, with no added GDL was the thinnest. However, there was a lot of variation in film thickness within a single treatment, e.g. the film with 41.67% added GDL.

The maximum force was the highest for the film with no added GDL and the film with the smallest quantity of added GDL (6.94%). The maximum force decreased as the quantity of added GDL increased, with the lowest maximum force obtained from films with 41.67% added GDL (about 50% less than the maximum force required to break a film strip with no added GDL). This was the same for force at break.

For stress, calculated by using the mean film cross sectional area the film with 41.67% added GDL gave the lowest value and the film with no GDL gave the highest value (almost 3 times higher than the lowest value). The same trend occurred for the stress, calculated by using the minimum film cross sectional area, except that there was no significant difference between the stress values of the films with 41.67% and 34.72% added GDL.

For stress at break, calculated with the mean film cross sectional area and stress at break, calculated with the minimum film cross sectional area, again the films with 41.67% added GDL gave the lowest values and the films with no added GDL gave the highest values (more than 3 times higher than the lowest values).

For strain (extensibility), there was no significant difference between the values for films with no added GDL, 6.94% and 13.89% added GDL. These films exhibited the lowest extensibility. These extensibility values were significantly different from the extensibility values of films where 34.72% and 41.67% GDL was added. The latter films were the most extensible. In the case of these last two samples, there were wide variations in strain within a single treatment.

Table 17: Effect of different GDL concentrations on the moisture barrier properties of kafirin films

Plasticizer (percentage relative to protein weight) GDL[a]	**Thickness mean (µm)**	**WVTR ($g/h/m^2$)[b]**	**WVP (gmm/m^2hkPa)[c]**
0	91a[d] (6)[e]	5.22a (0.37)	0.200a (0.026)
6.94	106ab (14)	5.60ab (0.53)	0.250b (0.042)
13.89	95a (16)	6.16bc (0.80)	0.241b (0.011)
20.83	94a (13)	6.41cd (0.30)	0.253b (0.045)
27.78	92a (7)	6.90d (0.66)	0.265bc (0.010)
34.72	112b (11)	6.25bcd (0.19)	0.293c (0.019)
41.67	110b (6)	6.37cd (0.26)	0.296c (0.009)

A constant amount (0.2 g) of LA was added together with the GDL to each film
[a] GDL = Glucono-delta-Lactone
[b] WVTR = Water vapour transmission rate
[c] WVP = Water vapour permeability
[d] Values with different letters in the same column are significantly different from each other at the 95% level
[e] Figures in parentheses indicate the standard deviation

In Table 17, the thicknesses of the films did not show great variation, though it seemed as if the thickest films are the ones with the most added GDL. Wide variations in film thickness within a single treatment existed, e.g. films with 6.94% and 13.89% added GDL. The greatest significant difference in WVTR existed between that of the control film with no added GDL and that of the film with 27.78% added GDL. The control film had the lowest WVTR and the film containing 27.78% GDL had statistically the highest WVTR (1.3 times higher than the lowest value). However, the WVTR of the films, containing 20.83%, 27.78%, 34.72% and 41.67% added GDL were all very close to each other, being practically the same.

The films with 34.72% and 41.67% added GDL had the highest WVP (about 1.4 times higher than the lowest value), with no significant difference existing between them. These films differed significantly from the control film (0% GDL), which had the lowest WVP of all the films with added GDL. In fact, the control film differed significantly from all of the other films with regards to its WVP.

Sensory evaluation of films with added GDL

When comparing the films with added GDL and the control with no GDL sensorically to a clear polythene bag, the kafirin films were found to be all relatively clear and transparent, yellow in colour with a mild odour. The surface texture tended to vary from smooth to an intermediate roughness as the amount of added GDL increased. This was especially true when compared to the control film, which had a very smooth surface. The flexibility of the films was not changed a great deal with the addition of GDL, and all the films were still relatively brittle.

4.1 Ultrastructure of kafirin films

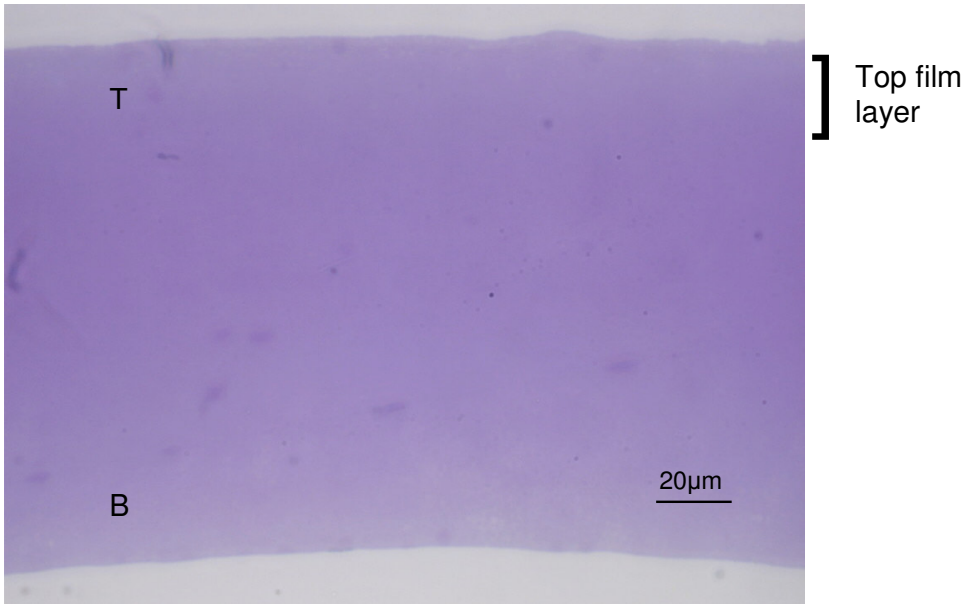
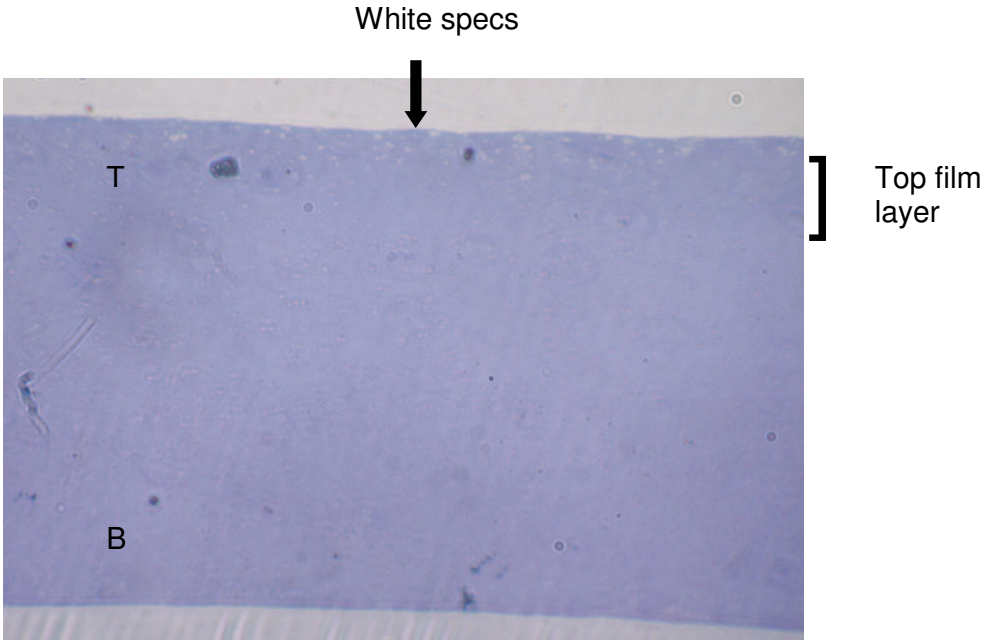
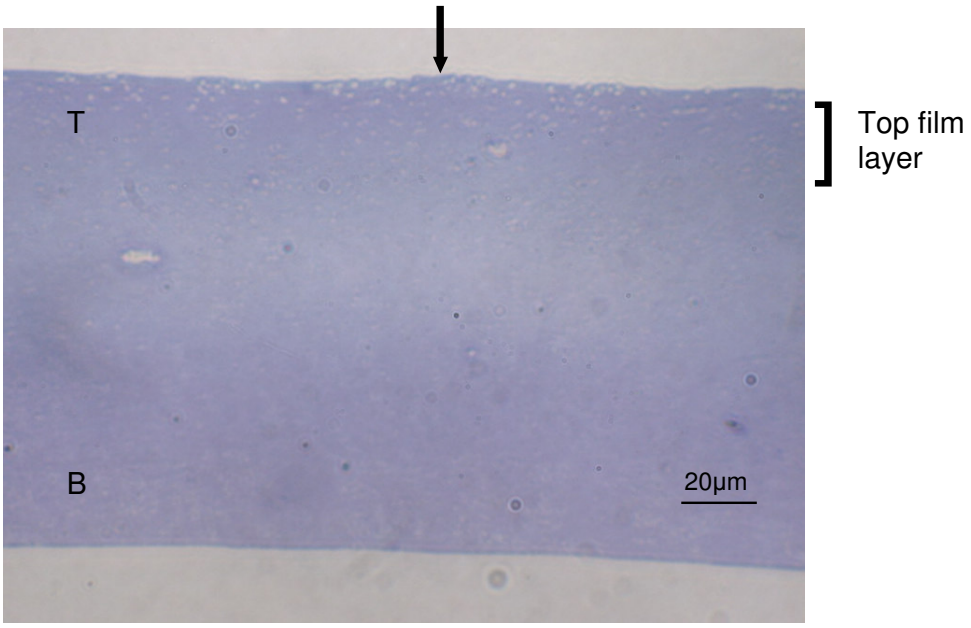


Figure 12: Light microscope images of kafirin films with different plasticizer combinations

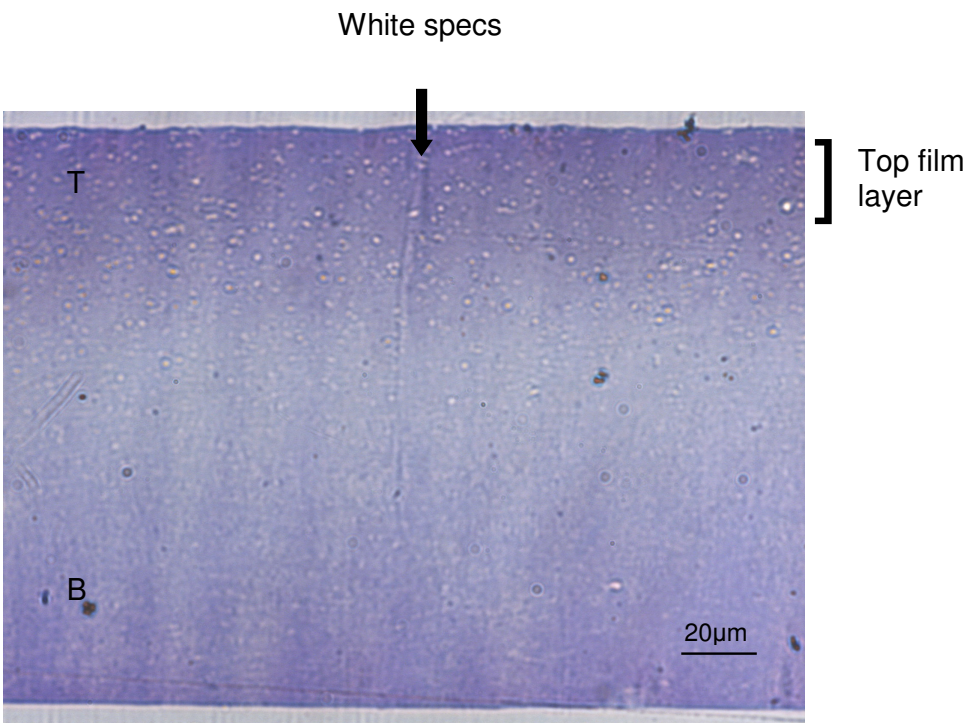
a. Micrograph of film with no added plasticizer



b. Micrograph of film with plasticizer combination G3.8, PEG3.8, LA3.8



c. Micrograph of film with plasticizer combination G8.0, PEG8.0, LA8.0



d. Micrograph of film with plasticizer combination G12.2, PEG12.2, LA12.2

In order to eliminate experimental error, many micrographs were taken and studied to make sure they were representative. The micrographs of the kafirin films, a to d are in increasing order of the plasticizer amount. In the film containing no added plasticizer (Fig. 12a), no white specks could be seen in the top layer of the film. The white specks increased as the plasticizer amount in the film increased (Fig. 12b-d), either by becoming more prominent in the top layer of the film, or by spreading through the middle section of the film. Most of the white specks were present in the top half of the film.

5. DISCUSSION

The discussion that follows will deal with two aspects: the methodology used for the experimental procedures and the effects of the different plasticizers on kafirin film properties. The discussion on the methodology will deal with the different assays used for determining the tensile and water vapour barrier properties of the kafirin films. It will give a brief overview on the determination of the glass transition and oxygen permeability properties of the films and the methodology used for microscopic analysis. The test conditions that could have had an effect on film properties, e.g. relative humidity and temperature will also be discussed. The section on plasticizer effects will discuss the choice of plasticizers, in other words, why G, PEG, LA, DATEM and GDL were chosen and tested as possible plasticizers for kafirin films. It will also deal with the influence of the different plasticizer combinations as well as the moisture absorbed by the films on the film properties, giving possible reasons for these effects. Each one of the film properties will be discussed individually: tensile-, glass transition (T_g)-, moisture- and oxygen transmission. Lastly, a model for plasticizer action will be presented to attempt to explain the findings.

5.1 Methodology

The experimental work in this project started in winter and went on right through until summer. Between winter and summer, the temperatures and the RH in the laboratory where the tensile properties of the films were determined varied a great deal because it was not controlled. The temperatures in winter can be as low as 14°C with a RH of 14%. In summer, temperatures of 30°C accompanied by a much higher RH (≈ 41%) can be expected. Temperature and RH are two factors that can influence the properties of protein films to a great extent (Gennadios *et al.*, 1993; Mujica-Paz & Gontard, 1997). Films prepared from hydrophilic proteins will absorb moisture from the atmosphere and this will affect the film properties. Gennadios *et al.* (1993) found that RH and temperature affected wheat gluten films more than films made of maize zein. Their explanation was that this was due to the fact that zein is more hydrophobic than gluten. As discussed, kafirin is also a very hydrophobic

protein (Wall & Paulis, 1978), somewhat more hydrophobic than zein (Duodu *et al.*, 2003), perhaps making it less prone to water absorption. The water absorbed by the kafirin films with no added plasticizers, stored at 50% RH, proved to be about 6.3%. In other words, despite the hydrophobic nature of kafirin, it will still contain some water, which will influence the film properties.

The plasticizers which were added to the kafirin films were hydrophilic, especially G (Cuq *et al.*, 1997) and PEG (Park *et al.*, 1994). When the individual plasticizers were stored at 50% RH, G absorbed 12.8% moisture, PEG absorbed 7.6% and LA did not absorb any moisture. PEG is said to be less hydrophilic than G, probably due to its longer carbon chain (16 carbons) in comparison to that of G (3 carbons). Coupland, Shaw, Monahan, O'Riordan & O'Sullivan (2000) found that G has a tendency to attract water, which had an additional plasticizing effect on the whey protein isolate films they were studying. As stated, the kafirin also had a certain amount of moisture associated to it. Due to the hydrophobic nature of kafirin, the moisture associated with the kafirin was very small in comparison to the moisture absorbed by each plasticizer. The plasticizers were the major cause for moisture absorption by kafirin films (Table 4). This happened because a controlled relative humidity environmental chamber was not available to test the films in. Therefore, this could also happen when the films are used in practice.

An example of how RH differences between winter and summer influenced the film properties is with the film containing the least amount of plasticizer, combination G3.8, PEG3.8, LA3.8. During times when the RH was higher, it was possible to test the film with the texture analyzer without it cracking when handled. During times when the RH was extremely low, it was practically impossible to handle these films without it cracking, leading to a very tedious analysis with many repetitions in order to obtain sufficient measurements.

A modified version of the ASTM D882-97 method (American Society for Testing and Materials, 1997) was used to determine the tensile properties of the films. The maximum force, the force required to break the film strips and

the distance each film was stretched were read from the computer. The variation in film thickness between different film strips due to the different amounts of plasticizer the different films contained, and the different amounts of absorbed water from the atmosphere with varying RH, could have influenced the force measurements. This variation was, however, eliminated in the calculation for stress (see section 3.4.4). Therefore, the variation in film thickness between treatments should not have had an influence on the results.

In order to minimize the possibility of external influences (RH and temperature) on film properties, films were conditioned after drying. They were held overnight in a desiccator, containing a saturated calcium nitrate solution. This environment provided a stable RH of 50%, ensuring that all the films were treated the same. This followed the procedure of Aydt *et al.* (1991) and Lai & Padua (1997). However, after conditioning, a time of up to 5 hours elapsed from taking the films out of the desicator and measuring the thickness until the tensile tests were finished. This might have been a cause for variation. The tensile data can therefore not be compared directly with values in the literature, since conditioning procedures and treatments differ. Notwithstanding this, trends can be compared.

Since moisture loss in food is one of the biggest causes of food quality deterioration (Potter & Hotchkiss, 1995), the water barrier properties of films are important when films are to be used as moisture barriers in a specific food application. The water vapour transmission of films refers to a film's ability to control the water vapour transport between the food system it is applied to and its surroundings (Gennadios, Weller & Gooding, 1994). For the determination of the water vapour barrier properties of free standing cast kafirin films, an adapted version of the "cup method" ASTM method E96-97 (American Society for Testing and Materials, 1997) was used. Instead of using a cup, Schott bottles (100 ml) were modified as described in the Materials and Methods section. Since the original method specifies the use of a cup of uniform diameter, there is some concern that the contours of the

bottle (broad at the bottom and narrow at the neck) might influence the water vapour transmission rate (WVTR).

What was of greater concern though, was the air gap between the underside of the film and the water surface. The gap was present to prevent contact between the water and the film. This air gap causes a stagnant air layer that is resistant to water vapour transmission. This can lead to an underestimate in WVP of between 5 and 46% (Gennadios *et al.*, 1994). For high water vapour-transmitting hydrophilic films, this error needs to be corrected, but since kafirin is very hydrophobic in comparison to most other proteins used for film making (Wall & Paulis, 1978), kafirin films should have lower transmission rates than other protein films. Also, even with the air gap increasing as the water evaporated, the water loss remained linear over time (Da Silva, 2003). For these reasons the air gap was not deemed critical.

Also, according to Gennadios *et al*. (1994), the stagnant air layer does not only exist under the film, but could also be present above the bottle or cup. This was avoided by the fact that a constant movement of air was provided by the fan of the fume cupboard for the entire test period (8 days).

Environmental factors, such as RH and temperature, can also affect the WVTR of films. Gontard *et al.* (1993) reported that due to an increase in film water activity, the WVTR of gluten films increased. A possible reason given for this was that with water absorption, the protein network swells, making it easier for water molecules to diffuse through the protein network. Therefore, with the differences in RH and temperature that occurred between winter and summer, films could have had different levels of absorbed moisture, leading to different values for the WVTR. Depending on the amount and combination of the plasticizers the films contained, the amount of moisture also differed among films (Table 4). The reason for this is that more hygroscopic plasticizers, e.g. G, would absorb more moisture from the atmosphere than others (Lawton, 2004).

The fact that the glass transition (T_g) and the oxygen permeability (OP) properties of the films were not determined in South Africa, but at SIK in Sweden, complicates discussing critically the methodology used. The films for these analyses were prepared locally by the standard procedures, packaged and sent to Sweden. The changes in temperature and RH that the films were exposed to during this time presumably had an impact on their measured properties. Due to the time that elapsed while the films were sent to Sweden, the films were also older when tested there than when tested in South Africa. After film production, one month passed before the T_g properties were determined and 2.5 months before finishing the OP determinations. This was a long time in comparison to the 24 hours that passed between film production and testing in South Africa. According to Park *et al.* (1994), Park *et al.* (1992) found that G, when used singly as a plasticizer tends to migrate from the film matrix over time. In this case, G was not used as a plasticizer alone, but it is not certain what effect time could have had on it and how its migration could have influenced the results of the T_g property- and OP tests.

For the OP tests, the environmental differences in RH and temperature were overcome by the fact that the films were preconditioned and tested at a constant RH of 50% and a temperature of 23℃ (Anker *et al.,* 2000). For the determination of the T_g properties of the films, films strips were also preconditioned at 50% RH and 23℃, ensuring that the initial conditions were the same. The conditioning step was followed by the application of a hydrophobic grease to prevent any moisture loss during testing (Stading, 1998). This actually ensured a stable environment, leading to comparable results since most other influential factors were eliminated and variability could therefore be only due to the differences in plasticizer.

The microstructural analysis of the films were repeated many times in order to make sure that the micrographs were an accurate representation of the actual structure of the films. Film strips were embedded in resin in order to be able to cut sufficiently thin sections (0.5 µm) (as reviewed by Afzelius & Maunsbach, 2004). Before sample embedding was used, attempts to study cut sections of 80-100 µm in thickness were not successful. The film sections were too thick

for the illuminating light to penetrate, leading to dark micrographs without sufficient detail. With embedding, very thin sections were possible but when film sections were only slightly too thick (> 0.5 µm), it was not possible to focus only on one plane. This made it difficult to distinguish between what was real and what was a consequence of the inability to focus on one plane.

During the embedding procedure, film strips were dehydrated by washing them in a series of aqueous ethanol solutions of different concentrations. Since the plasticizers G and PEG used in the films are hydrophilic (Di Gioia *et al.*, 1998) and therefore water-soluble, the washing step caused them to be washed out. The washing step was, however, necessary, since the osmium tetroxide used during fixation, tended to give images stained with dark specks if not washed out.

The interpretation of what exactly was seen on the micrographs of the plasticized kafirin films is very difficult and only speculations can be made. The literature published on protein film microstructure is very limited, but can be helpful in explaining the kafirin micrographs, especially those articles on zein film microstructure. Publications on the microstructure of different protein films include the following: yuba-films made from soybean protein (Okamoto, 1978), whey protein films (Anker *et al.*, 2000) and zein films (Lai & Padua, 1997; Lai et al., 1997; Yoshino, Isobe & Maekawa, 2000). The literature that has been published on the microstructural analysis of plasticized kafirin films is limited to the very recent research by Emmambux (2004).

5.2 Film properties as influenced by different plasticizer combinations

With regard to the choice of plasticizers, G, PEG 400 and LA were chosen for specific reasons and used in combination with each other to plasticize kafirin films. G and PEG are two plasticizers that are generally used for protein film plasticization, either alone or together in the same film system. For example in sodium caseinate films (Siew *et al.*, 1999), gluten and zein films (Aydt *et al.*, 1991; Gontard *et al.*, 1993; Park *et al.*, 1994; Lawton, 2004), whey protein films (Coupland *et al.*, 2000) as well as in kafirin films (Buffo *et al.*, 1997).

According to the review by Lawton (2002), LA is a very useful plasticizer for zein films and additionally, it has been classified as a primary zein solvent for zein. Since zein and kafirin are so similar in many ways (Shull *et al.*, 1991) a small amount of LA added during film manufacture should not only contribute to film plasticization, but also help to dissolve the kafirin. Kafirin is less soluble in 70% aqueous ethanol than zein, probably due to its more hydrophobic nature (Wall & Paulis, 1978). The choice of combining G, PEG and LA was based on previous experience in our laboratory (Da Silva, 2003; Taylor, 2003; Emmambux, 2004) where this combination was found to work effectively as a plasticizer for kafirin films.

Attempts to improve the three-plasticizer system (G, PEG and LA), mentioned in the previous paragraph, to a two-plasticizer system lead to including glucono-δ-lactone (GDL) and diacetyl tartaric ester of monoglyceride (DATEM) during film making. From preliminary studies done by the author, it was observed that LA was necessary to give clear films, in other words to help dissolve the kafirin protein. GDL, an acidulant (as reviewed by Berry, 2001), was chosen as a possible plasticizer to combine with LA. It proved to have good plasticizer properties that seemed to work in a kafirin polymer system (Personal communication, Ms S. Buchner, Food Scientist, Bio/Chemtek, CSIR, Pretoria). DATEM, on the other hand, is an emulsifier (Stauffer, 1996). DATEM has previously been used as a plasticizer in thermoplastic resins, made of maize gluten meal (Di Gioia & Guilbert, 1999). Therefore, it was thought worthwhile to try both an acidulant and an emulsifier in the kafirin films to determine if a two-plasticizer system was possible with either of them.

Water has been found to be a good plasticizer for zein (Madeka & Kokini, 1996) and because zein and kafirin are so similar (Shull *et al.,* 1991), it should also be a good plasticizer for kafirin. G and PEG are very hydrophilic in nature (Park *et al.,* 1994) and will absorb moisture from the atmosphere (Lawton, 2004). The absorbed moisture will have an additional plasticizing effect and therefore also needs to be taken into account.

When a plasticizer is added to a film, the film's tensile properties are changed. According to Banker (1966), the addition of a plasticizer will cause a reduction in film brittleness, will lead to a decrease in tensile strength and will make the film more flexible. When a plasticizer is added to a protein (external plasticization), the amount and type of plasticizer will determine its degree of flexibility (Sommer, 1985). As expected with plasticizer addition, the kafirin films with higher amounts of plasticizer required less force to deform and break them, but they were much more extensible than films with less plasticizer (Table 4). Films that contained very little plasticizer remained brittle despite the presence of the plasticizer, indicating that the amount of plasticizer was not sufficient for plasticization. Depending on the end use of the film, the amount of plasticizer clearly can be adjusted to influence the film properties to fit the use.

As for plasticizer type influencing the tensile properties of films, some seemed to be more effective than others. G appeared to have a greater effect on the extensibility of kafirin films in comparison to PEG and LA (Table 12). For the kafirin film strain (extensibility) to increase drastically, the G content of films would have to increase twice as much as that of PEG and LA. This is probably because G molecules are very small (MW 92) with a carbon chain length of only 3 carbons (Park *et al.*, 1994). Gontard *et al.* (1993) suggested that the reason why G was a good plasticizer for gluten films was because of its small size. According to them, the G molecules can easily be inserted between the protein chains, where it would interact with the protein chains through hydrogen bonding. This will reduce the original interactions between the protein chains as well as increase the distance between the chains – all of this will lead to more flexible films (Gontard *et al.*, 1993).

The plasticization action of G, as described above, could be a combination of the lubricity theory and the gel theory, both described by Sears & Darby (1982). The speculation that the small G molecules get inserted between the protein chains is an application of the lubricity theory. According to this theory, as a force is applied to a polymer, the plasticizer will act as a "lubricant" for the internal glide planes of the polymer chains. This makes it easier for the

polymer chains to glide over each other, lending flexibility to the polymer. However, this theory assumes that no or very weak bonding takes place between plasticizer molecules among themselves or with the polymer chains. According to Sears & Darby (1982), the gel theory states that a polymeric material consists of a three-dimensional gel, formed by polymers that are bound together through loose attachments that occur along the polymer chains. If the polymeric material is brittle, the points of attachment are close together and the cell dimensions are small. If a plasticizer, in this case G is added, it will break some of the attachments along the polymer chains, leading to less attachments. This will increase the flexibility of the structure (Sears & Darby, 1982). In other words, the G will break some of the existing bonds between the kafirin protein chains and form new ones, increasing the flexibility of the kafirin films.

According to Gontard *et al.* (1993), G formed mainly hydrogen bonds in wheat gluten films when reacting with the amide group of the amino acid glutamine. Glutamine is a polar amino acid with an amide group in its side chain (Campbell, 1999). Since kafirin is also rich in glutamine (Wall & Paulis, 1978), G could also form hydrogen bonds with the amide groups present. Therefore, as stated in the previous paragraph, one could say that G will be inserted between the kafirin polypeptide chains, where it will break some of the existing bonds between the chains and itself form new hydrogen bonds with the polypeptide chains.

As for PEG, Siew *et al.* (1999) suggested that due to its larger size, PEG might be prevented from interacting with some of the hydrophilic binding sites on sodium caseinate polypeptide chains. It cannot be inserted between polypeptide chains because of stearic hindrance between neighbouring side chains that will prevent it. PEG will therefore not be able to disrupt protein-protein interactions and is more likely to form cross-links between the polypeptide chains as well as interchain hydrophobic bonds. With kafirin plasticization, PEG was, however, found to be more significant in influencing film properties than G and LA with the principal component analysis (Table 13). Therefore, even though it is suspected that PEG cannot penetrate the

protein network to the same extent as G, it does seem to play a leading role in the overall plasticizing effect of the three-component plasticizer system used for kafirin films.

The role of LA as a plasticizer in this three-component plasticizer system is in doubt, since LA's influence on the films properties was not found to be significant (Table 13). It was, however, noticed that LA was necessary in order for kafirin to dissolve properly. This was supported by the fact that Taylor (2003) found LA to be a good primary solvent for kafirin. Therefore, it was concluded that apart from its influence on kafirin solvation, LA did not really play a role in kafirin film plasticization. This is in fact an anomaly, because according to Fennema (1996), even though a plasticizer is not always a true solvent, a true solvent for polymeric materials is always a plasticizer. This means that because LA is a solvent for kafirin, it has to play some role in plasticizing the kafirin films. The results (Tables 12 & 13), however, do not show this and it cannot be explained.

With the addition of DATEM, an emulsifier, in combination with LA, no significant effect was observed on either the force needed to deform and break the films, or on the film extensibility (Table 14). The tensile strength of the films did not decrease with increasing plasticizer content, nor did the extensibility of the films increase as expected. In fact, DATEM did not seem to have any plasticizing effect on kafirin films. The kafirin films made with DATEM also appeared to have phase separation on the film surface, especially with higher concentrations of DATEM. It seemed as if the DATEM could not be sufficiently incorporated into the protein matrix during filmmaking.

Di Gioia & Guilbert (1999) used DATEM as an amphiphilic plasticizer in maize gluten meal resins and found it to have a good plasticizing effect. The fact that it was found to be an effective plasticizer in resin films and not in cast films may be due to the fact that the DATEM could not be dissolved sufficiently during film casting. When comparing kafirin film casting and thermoplastic resin film production as done by Di Gioia & Guilbert (1999), there was not much difference in production temperature (70℃ versus 80℃) or stirring

(magnetic stirrer versus a counter-rotating batch mixer) that could have lead to the difference in DATEM solvation. However, during film casting, 70% aqueous ethanol was used to dissolve the kafirin, LA and DATEM but not in resin film production. Since the DATEM molecule contains a monoglyceride component (diacetyl tartaric ester of monoglyceride), it could be that it is too hydrophobic to dissolve properly in the aqueous ethanol. DATEM and aqueous ethanol seemed to be incompatible.

GDL proved to be successful in the plasticization of cast kafirin films. Film strength decreased and extensibility increased with increasing GDL content, as would be expected from a good plasticizer. GDL has been used previously in soy-protein isolate films by Park *et al.* (2001). It was found to cause a great increase in film extensibility as the GDL content increased. GDL has a molecular weight of 178, about twice that of G (MW 92). Therefore, from a molecular point of view, it should easily be inserted between protein chains to plasticize the polymer, following the same pattern as G. Park *et al.* (2001) stated that GDL binds to soy proteins mainly through hydrogen bonding. It is possible that the GDL molecules were inserted between the polypeptide chains where it formed hydrogen bonds and increased the interchain distance, as was proposed for G by Gontard *et al.* (1993). This lead to plasticization.

Phase separation was visible in some of the films, but could not be related to increased levels of GDL. Therefore it can be concluded that the method of GDL incorporation into films was not the most effective method, thus leading to much variability.

Evans and Manley (1941) stated that for a substance to be a good solvent for zein, the presence of an -OH, $-NH_2$, $-CONH_2$ or a –COOH group is important. Taylor (2003) showed LA and glacial acetic acid to be good solvents for kafirin, both of them containing the –COOH group. When dissolved in water, GDL will hydrolyze and form gluconic acid (reviewed by Berry, 2001). As an organic acid, it also contains the –COOH group. It may therefore be that GDL aids in protein solvation due to the formation of gluconic acid in the presence

of aqueous ethanol. However, when used in combination with LA in a two-component plasticizer system, either GDL or LA or both must play a role in plasticization apart from protein solvation, since there was a clear plasticizing effect.

Moisture absorption leads to plasticization. Lawton (2004) found the elongation of plasticized zein films to increase and film strength to decrease with increasing storage RH. As already stated, this was also illustrated by kafirin films with very little added plasticizer (plasticizer combination G3.8, PEG3.8, LA3.8). This film was almost too brittle to be tested during low RH times, but its flexibility increased when the air RH increased. The absorbed moisture therefore increased film flexibility and decreased film brittleness, acting in exactly the same way an added plasticizer would have. In the films, each plasticizer absorbed a certain amount of moisture at 50% RH (Table 4). The plasticizers were very significant contributors of moisture to the films. Thus, the more plasticizer a film contains, the more moisture it will absorb. This was clear from the principal component analysis performed (Table 13), where moisture content showed a very significant positive correlation with the plasticizers, G and PEG, confirming the statement that moisture content will increase with increasing plasticizer amount. The highly significant correlations, both positive and negative, between moisture content and other film properties indicate that moisture can be classified as the “plasticizer” that influences film properties the most.

One of the most important functions of a plasticizer is to lower the glass transition temperature (T_g) of the polymer it is added to (Sears & Darby, 1982). Glass transition has been defined as the point where a material changes from a brittle glass to a rubbery solid (Gontard *et al.* 1993). According to Sears & Darby (1982), a plasticizer will lower the temperature where this transition takes place. As expected, the lowering of the T_g of a material by a plasticizer was clearly shown by the differences in the T_g between the kafirin films containing high amounts of plasticizer in comparison to the films containing very little plasticizer. The more plasticizer a film contained, the lower the T_g of the film and *vice versa* (Table 5). The highly

significant negative correlation that existed between the T_g intersect and the T_g E"peak with the plasticizers, G and PEG (Table 13) also confirmed that the T_g would decrease as the amount of plasticizer increased.

Sears & Darby (1982) attempted to explain the relationship between plasticizers and T_g through the use of the free volume theory. According to this theory, the plasticity of a material depends on the amount of free volume that exists between its molecules. The addition of a plasticizer will cause the intermolecular free volume to increase through different mechanisms, as discussed in the literature review (section 2.3.3.1). In this particular case, through external plasticization, the manner of increasing the free volume in the kafirin polymer was through the inclusion of a substance with a lower molecular weight (Sears & Darby, 1982). Even though the molecular weight of G (MW 92) was much lower than that of PEG (MW 400), it seemed as if they contributed more or less equally to the depression of the T_g of kafirin films (Table 12). However, Lawton (2004) found that the plasticizer efficiency in lowering the T_g of zein films increased with plasticizer molecular weight, rendering PEG more efficient than G. Whether this was true for kafirin films is not very clear, but PEG was found to be more significantly negatively correlated with the T_g intersect and T_g E"peak of films than G (Table 13). This is an indication that PEG might have contributed more to the lowering of the T_g of kafirin films. Of all the plasticizers, though, moisture seemed to be the most influential on the abovementioned T_g properties, being the most significantly correlated.

At temperatures above the T_g of a specific material, the molecules of the material will have more energy to move, creating more free volume, which in turn causes the material to be more flexible. When a plasticizer is added, it will lower the T_g. Above this lowered T_g, the molecules will similarly have more energy to move. This creates more free volume and causes the material to be in a more flexible state at a lower temperature than without the plasticizer (Sears & Darby, 1982). One would have thought this to be the reason why the kafirin films without or with very little plasticizer were brittle at ambient temperature and the kafirin films with more plasticizer added were more

flexible at the same temperature. Without plasticizers, the T_g of the kafirin films might be above ambient temperature, causing the film to be in a glassy state at ambient temperature. With added plasticizer, the film T_g might have been lowered to below that of ambient temperature, causing the kafirin films to be in a rubbery state at room temperature. This was, however, not true in respect of the results actually obtained for the kafirin films, since the temperatures where the plasticized films changed from the glassy state to the rubbery state (T_g) (40 - 75°C) (Table 5) was much higher than ambient temperature.

Explanations for the action of the plasticizers on the glass transition properties of kafirin films can be drawn from the free volume theory, but it should not be forgotten that in this case, what is being dealt with was a composite material. The films were mixtures of a solid (kafirin) and a liquid (plasticizer). In some cases the plasticizer was present in a very large percentage, constituting a considerable part of the film, e.g the combination G12.2, PEG12.2, LA12.2 adds up to a total of 36.6% plasticizer as a percentage relative to protein weight. The absorbed moisture for this particular combination amounts to an additional 8.8% relative to protein weight. Therefore, 45.4% of the film's total composition is liquid. The theory may therefore not be totally applicable.

In general, due to the hydrophilic nature of proteins, protein films do not have good water vapour barrier properties (Krochta, 2002). The oxygen barrier properties of protein films on the other hand, are good at low or intermediate RH, making them useful as coatings for products that are oxygen-sensitive. As previously stated, zein and even more so kafirin are more hydrophobic than most proteins (Duodu *et al*., 2003), making them better prospects as moisture barriers than any other commonly available proteins. However, the addition of hydrophilic plasticizers such as G and PEG to the films will have a negative effect on the moisture barrier properties of the films. The WVP and WVTR of kafirin films showed an increasing trend as the plasticizer content of the films increased. Kafirin films with the lowest plasticizer content proved to have the best water vapour barrier properties, and as the amount of plasticizer increased the barrier properties deteriorated (Table 6). Park *et al.*

(1994) showed that the water vapour permeability (WVP) of both gluten and zein films increased with increasing plasticizer content (G and PEG), similar to what was found for kafirin films. Also, according to Park *et al.* (1994), Park *et al.* (1992) found the oxygen permeability (OP) of gluten and zein films to increase with an increasing G concentration. It was therefore also to be expected for kafirin films with higher plasticizer concentrations to have a higher OP and films with lower plasticizer concentrations to have a lower OP (Table 7).

As already explained in the T_g section of this discussion, according to the free volume theory the addition of a plasticizer to a polymeric system, in this case the kafirin protein film is a way to increase the free volume in that system (Sears & Darby, 1982). This causes the films to be more flexible because there is more space for the protein chains to move. But, this increase in space between the chains might make the system less dense, making it easier for water vapour and oxygen to escape through. Gontard *et al.* (1993) suggested that the reason why gluten film permeability increased with G addition, is due to the structural modification brought about by the plasticizer. They suggested that due to the protein-plasticizer interactions, the space between the polypeptide chains increases, leading to a less dense structure. This suggestion made by Gontard *et al.* (1993), previously explained in this discussion as a combination between the lubricity theory and the gel theory, could also be seen as an application of the free volume theory to explain the increased permeability. The increased distance between the polypeptide chains due to the formation of plasticizer-protein bonds would result in an increase in free volume, probably making the films more permeable. If this is so, in the case of kafirin films, with the plasticizers G and PEG positioned between the kafirin polypeptide chains, the distance between the chains would also increase. This would cause the system to have more free volume, rendering the kafirin network less compact. This would make it possible for water vapour and oxygen molecules to move through the film more rapidly.

The water vapour barrier properties and the oxygen barrier properties were significantly correlated to the G, PEG and moisture content of the films (Table

13). One can conclude that the more plasticizer present in the films (moisture is also classified as a plasticizer), the more permeable the film would be. It is significant that LA did not seem to influence the permeability of the films (Table 13).

Park *et al.* (1994) found that as the ratio of G to PEG in gluten and zein films decreased, the WVP of both films decreased. In other words, G had the greatest effect on increasing the permeability of the films. The reason given for this was that G is more hydrophilic than PEG. Due to its more hydrophilic nature, G would promote the adsorption and desorption of water molecules, increasing the water vapour transmission rate (WVTR) through the films (Gontard *et al.*, 1993). Similarly, it was clearly visible that G had to decrease by more than 50% in order for the moisture barrier properties of kafirin films to improve (Table 12). PEG also needed to decrease to get the same effect but not to the same extent as G. LA, however, seemed to have very little or no effect. Thus, it seemed as if LA had very little or no influence on the barrier properties of kafirin films. As discussed before, LA's function is probably mainly to assist in kafirin solvation.

As previously discussed, when DATEM was used as a plasticizer, no effect on the tensile properties of kafirin films was observed (Table 14). DATEM was therefore not considered to be a plasticizer for kafirin films. Similarly for the barrier properties of films plasticized with DATEM (Table 15), none of the expected plasticizer effects were observed. There was no increase in the WVTR of the kafirin films with an increasing DATEM content, and no clear conclusion could be drawn from the WVP data. As previously explained, the reason why DATEM is not a good plasticizer for cast kafirin films is suspected to be its incompatibility with aqueous ethanol. DATEM was observed not to dissolve during film casting and it is suspected that it is too hydrophobic to dissolve in aqueous ethanol due to the monoglyceride component in its molecular structure. DATEM could therefore not act as a plasticizer and increase the free volume between the polypeptide chains. This is a possible reason why film permeability was not changed with DATEM addition.

With regard to the barrier properties of the kafirin films plasticized with GDL, a clear increase in the WVP and the WVTR of the films could be observed as the GDL concentration increased (Table 17). As already explained, the addition of a plasticizer, in this case GDL, causes the free volume in a polymeric system to increase, bringing about flexibility because free volume permits the movement of polymer molecules (Sears & Darby, 1982). The increase in free volume might lead to a less dense polymer structure, bringing about the increase in permeability. Therefore, even though a permeability increase is not a positive attribute brought about by plasticization, GDL does exhibit good plasticizing properties, making it a good possibility for further plasticizer research on kafirin films.

The OP of the kafirin films, plasticized with GDL was not investigated, but from the data obtained on the moisture barrier properties, one could predict its behaviour towards oxygen permeability. As previously discussed, the kafirin films' moisture barrier properties deteriorated as the amount of GDL increased (Table 17). Based on this, the assumption can be made that GDL plasticized films will also have an OP that will increase with plasticizer content.

As can be seen, one can conclude that plasticizer action will influence film properties both positively and negatively. One could add a plasticizer in order to improve the film's mechanical properties, but the barrier properties of the film would deteriorate. In other words, one will have a film that is easy to apply and to handle, but it may not be able to prevent moisture from escaping the product. Whether the influence of a plasticizer on a film is positive or negative, depends however upon the application of the film.

In this research, kafirin was used for film production as a possible solution for the increase in permeability brought about by plasticizer addition. It was proposed that due to the fact that kafirin is more hydrophobic than any other possible protein alternative (Duodu *et al.*, 2003), it might have had an opposing effect to the increased permeability. However, kafirin's effectiveness was inhibited because of the use of hydrophilic plasticizers, i.e. G and PEG. The solution to this most probably lies with the use of one or a combination of

less hydrophilic plasticizers (e.g. fatty acids) to plasticize films made from a hydrophobic protein such as kafirin. Lai & Padua (1997) produced cast zein films, plasticized with oleic acid (C18:1), but microstructural images of the films appeared to show phase separation between the zein and the oleic acid. When used as a plasticizer in kafirin films, phase separation is probably also going to occur, but further research is necessary to confirm this.

GDL proved to be quite effective in plasticizing the cast kafirin film (Table 16), but with much less detrimental effects on its barrier properties (Table 17). When comparing the WVP of films plasticized with GDL (Table 17) to films plasticized with a combination of G, PEG and LA (Table 6) at almost equal plasticizer concentration (percentages relative to protein weight), it is clear that GDL films had better water vapour barrier properties. For example, plasticizer combination G12.2, PEG12.2, LA12.2 (36.6% plasticizer in total) had a WVP of 0.360 gmm/m^2hkPa, and with an almost equal amount of GDL (34.72%) the WVP of films was only 0.293 gmm/m^2hkPa (only 81% of the WVP of the film with plasticizer combination G12.2, PEG12.2, LA12.2). Cuq *et al.* (1997) concluded that the smallest and most hydrophilic plasticizers are generally the most effective plasticizers. As already stated, GDL has a molecular size (MW 178) about twice the size of G (MW 92). It is therefore also a small molecule, also hydrophilic but it is non-hygroscopic (Berry, 2001). G is very hygroscopic and will increase water absorption by the films (Lawon, 2004). Therefore, in contrast to G, GDL will not attract as much water as G. The non-hygroscopic character of GDL will not necessarily reduce the amount of water let through the film, the hygroscopic character of G might just be promoting it. This probably accounts for the big difference in barrier properties at equal plasticizer concentrations. This explanation is not definite and the situation is probably much more complex than stated, leaving much room for further research.

What causes a bit of a contradiction is the fact that PEG also seemed to play a major role in influencing the oxygen barrier properties of kafirin films. PEG needed to increase more substantially in comparison to G and LA in order for the OTr of films to increase (Table 12). Park & Chinnan (1995) reported that

the gas permeability of cellulose films increased as the concentration of PEG increased. Even if data on cellulose films may not be directly relevant for protein films, their speculation that the reason for this phenomenon was the length of the carbon chain of the PEG molecule (16 carbons) may be pertinent. If the PEG molecules were to be inserted between the protein chains, its longer carbon chain would create more free volume between the chains (Sears & Darby, 1982). This could contribute to a less dense polymer structure, letting through more oxygen. The fact that PEG was more significantly positively correlated to OP and OTr of kafirin films than the other plasticizers, G and LA (Table 13), supports this.

According to Park *et al.* (1994), G has a tendency to migrate from the protein polymer when used alone as a plasticizer. They reported that Park *et al.* (1992) found the surface of zein films becoming greasy within a matter of hours due to G migration. Another sign of G migration was that films became brittle again, losing their flexibility over time. In the light of this, it can be speculated that the white specks visible on the micrographs of the kafirin films were where plasticizer droplets were before washing. The droplets are in fact “pools” of plasticizer, which formed when plasticizer molecules coalesced with migration. The fact that the white specks increased with plasticizer content supports this theory. All of the specks were concentrated in the top half of the film, leading to the suspicion that some of the plasticizer migrated to the film surface over time. That these specks can be linked to G alone or to a combination of the three plasticizers is not possible at this stage and leaves room for more research. Tillekeratne & Easteal (2000) stated, however, because PEG is a larger molecule, it will not migrate once it is inserted between the protein chains due to the formation of strong hydrogen bonds with the polypeptide chains. This was supported by Park *et al.* (1994), stating that the long carbon chain of PEG 400 (16 carbon atoms) in comparison to that of G (3 carbon atoms) causes greater stability. Since no white specks could be seen in the bottom half of the kafirin film and all three plasticizers were water-soluble, leaving the specks when dissolved, it could be concluded that all of the plasticizers migrated to some extent. It is however a possibility that some of the plasticizer molecules that did not migrate and coalesced, still

remained in the bottom half of the film but it was not visible on the kafirin film micrograph. Due to the observed plasticizer migration, it is speculated that plasticizer action is mainly due to the increase in free volume when inserted between protein molecules and that the formation of bonds with the protein molecules only plays a minor role in plasticization.

It is also possible that the white specs, seen on the micrographs were not formed during plasticizer migration and coalescence over time, but were rather due to phase separation during film drying, when the kafirin and plasticizers were still in solution. This is believed to be so because plasticizer migration is a slow event and separation due to a density difference is faster. As the film dries, the plasticizer movement slows down because they become more fixed in the protein network. The white specs could therefore represent the positions occupied by the plasticizers in the protein network before washing.

5.3 A model for plasticization

To explain plasticizer action in a polymeric system like kafirin protein films, the model constructed by Argos *et al.* (1982) for zein structure is a good starting point. Although this model is only speculative, it has been the basis for a few graphical explanations regarding kafirin intermolecular binding (Emmambux, 2004; Personal communication: Prof. P.S. Belton, Professor in Chemistry, University of East Anglia, UK). The original model was proposed for zein structure, but since zein and kafirin are similar in so many ways (Shull *et al.*, 1991), this model can be applied to kafirin protein structure as well.

The model proposed by Argos *et al.* (1982) has already been discussed in detail in section 2.2.1. But just to summarize, the rod-shaped zein molecules will aggregate due to the hydrogen bonds and van der Waals interactions between the polar and hydrophobic residues along the neighbouring helices. The interactions between the polar glutamine (Q) molecules at the cylindrical caps will cause the zein cylinders to stack. With the addition of a plasticizer, as already discussed, the plasticizer molecules will be inserted between the

polymer molecules, in this case kafirin protein molecules, where hydrogen bonds will be formed between the plasticizer and protein molecules (Gontard *et al.*, 1993). This will increase the distance between the protein molecules, causing the free volume to increase (Sears & Darby, 1982). This brings about an increase in flexibility, due to the increased space for movement.

Based on the model by Argos *et al.* (1982), a possible graphical explanation for plasticizer action on kafirin is proposed (Figure 13).

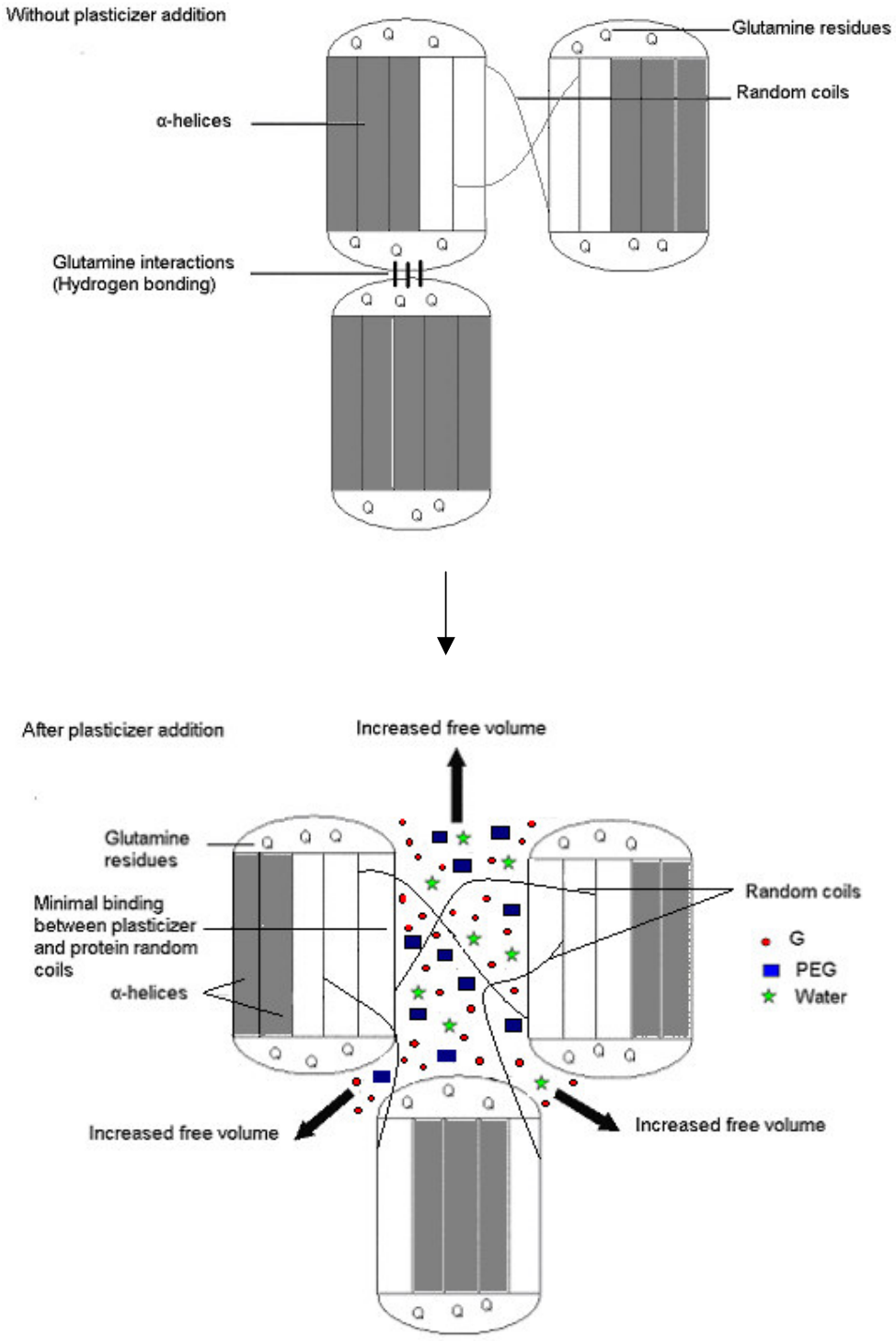


Fig 13: Proposed model for plasticizer action, based on the models constructed by Argos *et al.* (1982), Emmambux (2004) and Belton (Professor in Chemistry, University of East Anglia, UK, Personal communication).

According to Emmambux (2004), the β-sheet conformation of kafirin protein is broken down during film making (high temperature and vigorous stirring) to form random coils. The random coils provided binding sites where tannins could interact. Therefore, the random coils could also provide binding sites where plasticizers could interact. According to Siew *et al.* (1999), due to its size, PEG is more likely to promote hydrophobic intermolecular bonding between the polypeptide chains, forming cross-links between them. It was, however, concluded from the micrographs of plasticized kafirin films that the plasticizers migrated from the film matrix over time, meaning that the majority of plasticizer did not bind to the protein coils. This supports the theory that plasticizers are only inserted between the protein molecules, increasing the free volume, leading to more flexibility. According to Emmambux (2004), the heat also probably causes the hydrogen bonds between the glutamine residues to be broken down. This might also make it easier for plasticizers to move in between kafirin molecules, also leading to more free volume.

Lai *et al.* (1999) proposed a model for zein resin and cast films, plasticized with oleic acid. The insertion of plasticizer molecules between the α-helices of zein proteins was a possibility due to an indication of smaller zein interchain spacings in the absence of oleic acid. This was, however, not stated with certainty and therefore plasticizer action between α-helices is not indicated in Figure 13.

The model attempts to illustrate the above-mentioned concepts, showing the α-helices of the protein molecules, the random coils and the plasticizer molecules between the kafirin molecules. G, PEG and water are shown in the figure. LA was not included since it was concluded that LA did not partake in the plasticization of kafirin films, but rather in solvating the kafirin. The increase in space between the kafirin molecules, brought about by the plasticizers is also illustrated, indicated as an increase in free volume.

6. CONCLUSIONS AND RECOMMENDATIONS

G and PEG are plasticizers for cast kafirin films. Plasticizer content has a definite effect on kafirin film functional properties. The added plasticizers, as well as the absorbed moisture cause a decrease in film strength and an increase in strain. The T_g of the films decreases with increasing plasticizer content and the moisture and oxygen barrier properties of the films are reduced as the plasticizer content increase. Due to the effect the plasticizers have on film properties, the application of the films will inevitably be determined by the amount of plasticizer it contains. This means that a flexible film, as a result of a high plasticizer content, will not be suitable for application to something requiring a crunchy texture, like nuts. In contrast, a brittle film, with very little plasticizer will not be suitable for application to something soft and juicy, like a pear. Therefore, depending on the final application for the film, the film can be adapted to suit the purpose through adjusting the plasticizer content.

Moisture is a very influential plasticizer in kafirin films. Depending upon the amount of plasticizer present in the film and the RH of the film surroundings, the plasticizers absorb a certain amount of moisture, which influences the film properties just as do the added plasticizers. G and, to a lesser extent, PEG are responsible for moisture absorption from the atmosphere, and it is therefore recommended that less hydrophilic substances are researched as possible plasticizers for kafirin films, for example fatty acids.

Even though LA was expected to be a good plasticizer for kafirin, it did not prove to have any plasticizing effect on kafirin films. LA seems only to play a role in kafirin solvation during films casting. It is, however, clear that in the plasticizer combinations investigated all three substances G, PEG and LA are necessary to plasticize the kafirin films, and therefore, LA does play an important role.

DATEM, an emulsifier, is not a plasticizer for kafirin films, probably due to its incompatibility with aqueous ethanol, the kafirin solvent. GDL, an acidulant, is

a good plasticizer to kafirin films. GDL has the same plasticizing effects on kafirin films as G and PEG, but when compared to them at similar percentages relative to protein weight, GDL has a less detrimental effect on the WVP and the WVTR of the films. Thus, it is recommended that further research should be done on the mechanism of action of GDL, since it is not understood how GDL plasticizes the films.

From the plasticizer migration, observed by microscopy of the kafirin films, it is speculated that plasticizer action is mainly due to an increase in the free volume when the plasticizer is inserted between the protein molecules, and only to a lesser extent to the linking with proteins. Therefore it is recommended that a more stable plasticizer system should be researched for kafirin plasticization. This means that a plasticizer must be found that is able to link to the proteins to form a more permanent structure, leading to less migration. An alternative is to form kafirin resin films instead of cast kafirin films, as already done for zein films. If this proves to be possible, plasticization by means of fatty acids can probably be used.

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