Improvement in the size and antioxidant activity of kafirin microparticles by treatment with sorghum polyphenols

By

Juliet Muronzwa

Submitted in partial fulfilment of the requirements for the degree

MSc

Food Science

In the

Department of Food Science

Faculty of Natural and Agricultural Sciences

University of Pretoria

South Africa

July 2013

DECLARATION

I hereby declare that the dissertation herewith submitted at the University of Pretoria for the award of MSc (Food Science) degree is my work and has not been submitted by me for a degree at any other university or institution of higher education.

Juliet Muronzwa

ABSTRACT

Improvement in the size and antioxidant activity of kafirin microparticles by treatment with sorghum polyphenols

By

Juliet Muronzwa

Supervisor: Prof John RN Taylor

Co-supervisor: Dr Janet Taylor

Microparticles (KEMs) made from the sorghum prolamin protein, kafirin, have internal vacuoles. Hence, they have potential as delivery vehicles for nutraceuticals. However, their physico-chemical properties need to be improved for this application. The influence of kafirin extracted from white tan-plant and red non-tannin sorghum types of 81% and 84% protein content respectively and the rate of water addition on the formation of KEMs from kafirin in acetic acid solution by coacervation on their morphology was investigated. A water flow rate of 1.4 and 0.7 ml/min during coacervation using 81% kafirin resulted in spherical-shaped KEMs between 1 and 10 µm in diameter and vacuoles up to 2 µm. KEMs made with 84% kafirin at a flow rate of 0.7 ml/min were large and oval-shaped with an average length and width of 43 and 21 µm respectively and numerous vacuoles up to 3 µm. At a flow rate of 1.4 ml/min, the KEMs were oval-shaped with larger vacuole sizes (5 µm), a length and width of 91 and 30 µm respectively. However, SDS-PAGE indicated that neither the source of kafirin, nor the conditions of microparticle preparation had an effect on KEMs protein molecular size.As the presence of phenolic compounds in the kafirins might have been responsible for the differences in KEMs morphology, the effect of sorghum-derived polyphenols (extracted from condensed-tannin and non-tannin black sorghum brans) on the physico-chemical properties of KEMs was then investigated using 81% kafirin. Aqueous condensed tannin (10.1 mg CE (catechin equivalent)/100 mg extract) and black non-tannin (4.6 mg CE/100 mg extract) extracts in varying concentrations, were substituted for the water used for coacervation. KEMs made with condensed tannin extracts were oval-shaped and much larger, than control KEMs ranging from 20 to 400 µm, with rough surfaces and enlarged vacuoles. The enlarged vacuoles

were probably due to more air being trapped within the particles during formation. However, KEMs made from non-tannin phenolic extracts were smaller and spherical with average diameters up to 18 µm. Tannins are known to bind strongly to kafirin through hydrogen and hydrophobic bonds, which probably resulted in the larger microparticles. The KEMs made from condensed tannins also had high antioxidant capacities compared to KEMs made from non-tannin phenolic extracts, attributed to tannins being more potent antioxidants. Thus, condensed tannin extracts are the most beneficial as they contributed towards the antioxidant activity of the KEMs, resulting in the development of innovative KEMs with added antioxidant benefits and enlarged size.

DEDICATION

In loving memory of my late mother Mrs Mary Muronzwa

To my family especially my son Ben Anashe

And my husband Lenny

ACKNOWLEDGEMENTS

I owe sincere thankfulness to my supervisor, Prof John Taylor, who made me believe in myself. My research would not have been possible without his excellent guidance, positive criticism throughout and steadfast encouragement.

Many thanks to my co-supervisor, Dr Janet Taylor, for her outstanding supervision, support and kind concern. She truly polished my analysis skills in the lab with her technical support and skills. To Alan Hall and Chris van der Merwe for their technical help with microscopy.

I am grateful to the Institute for Food, Nutrition and Well-being for their financial assistance towards my study.

I would also like to thank the academic and non-academic staff, fellow postgraduate students in the Department of Food Science, University of Pretoria, who contributed in any shape or form to the completion of my studies. Special thanks to Joseph Anyango for his help and support.

Last but not least, my friends, family, husband and son, Ben ‘the kafirin baby’; for their support and encouragement. Many thanks to the one above all, God, for giving me strength through it all, even when l wanted to give up and throw in the towel, thank you dear Lord.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1 INTRODUCTION

Polymer-based particulate systems have attracted considerable attention as active molecule delivery devices (reviewed by Chen, Remondetto and Subirade, 2006). Known as microspheres or microparticles, these are defined as particles less than 1000 µm in size consisting of solid, liquid or gaseous materials encapsulated within their polymeric matrix or adsorbed or conjugated on to the surface (reviewed by Allèmann, Leroux and Gurny, 1998).

The biomedical sectors mostly use microparticles as carriers for drugs, to protect and transport to target functions (Peppas, Buresa, Leobandung and Ichikawa, 2000). However, in food applications, several purposes are targeted: i) the protectionof the encapsulated product against damage caused by external conditions such as light or heat during processing and storage in the food matrix, and ii) the separation of reactive species (Loveday and Singh, 2008). Further, an important benefit of microparticles is due to their ability to control the release of incorporated ingredients and deliver them to a specific target at a suitable time (Fernandez, Torres-Giner and Lagaron, 2009).

Microencapsulation is a technology that is used to encapsulate compounds inside microparticles (Dubey, Shami and Rao, 2009). Various techniques such as solvent extraction, spray drying and coacervation have been used for encapsulation (reviewed by Freitas, Merkle and Gander, 2005). Many synthetic polymers despite their successful elaboration have not been used in food applications as delivery systems which require generally regarded as safe (GRAS) substances. Thus interest in developing microparticles from food proteins as delivery systems has grown (reviewed by Chen et al., 2006).

As reviewed by Nesterenko, Aric, Silvestre and Durrieu (2013), proteins extracted from animal (whey, gelatine and casein) and plant (soy, pea and cereal proteins) derived products are widely used for encapsulation of active substances. Proteins because they are natural polymers, present several advantages: biocompatibility, biodegradability, good amphiphilic and functional properties. Plant proteins are also known to be less allergenic compared to animal derived proteins (Jenkins, Breiteneder and Mills, 2007). Thus, protein-based microparticles have found increasing applications in the food industry because they can be precisely designed for use in many food formulations and virtually any ingredient

can be encapsulated whether hydrophobic, hydrophilic or microbial (reviewed by Chen et al., 2006). There is growing interest in encapsulation of bioactive compounds, particularly polyphenols because of their associated health benefits (Fang and Bhandari, 2010).

The sorghum storage protein, kafirin, has been shown to have potential as a delivery system for phytochemicals (Taylor, Taylor, Belton and Minnaar, 2009b). Kafirin microparticles (KEMs), prepared by phase separation from a solution of kafirin in glacial acetic acid was shownto have a large internal surface together with the ability to form open structures (Taylor, Taylor, Belton and Minnaar, 2009a) and thus have the potential to be used as encapsulation agents for phenolics (Taylor et al., 2009b). According to Matalanis, Jones and McClements (2011), the functional performance of a protein microparticle depends on its structure- size, shape and internal morphology. However, in most cases the initial structure needs to be manipulated to improve on its functional properties. Therefore this project sought to investigate physical and chemical methods to control the structure of KEMs.

2 LITERATURE REVIEW

In this review, the concept of microparticles is explained, with particular focus in protein microparticles and why they are gaining interest in food applications. The principles involved in the formation of protein microparticles are also discussed as well as the various technologies or operating processes used to prepare the microparticles. The application of protein microparticles in microencapsulation is also discussed, especially as delivery vehicles for nutraceuticals. Specific reference is made to sorghum polyphenols as a nutraceutical that requires to be microencapsulated due to their antioxidant and health-promoting activities. A comparison is then made between animal-based and plant-based protein microparticles and why plant-based protein microparticles are preferable, in particular, sorghum protein, kafirin. Briefly, the characteristics of kafirin are explained and the microparticles made from kafirin. Lastly, the functional properties of protein microparticles relevant to their application are described. Physical and chemical methods of cross-linking protein microparticles, improving their functional properties are also discussed.

2.1 MICROPARTICLES

2.1.1 Definition

As reviewed by Nesterenko et al. (2013), microparticles are generally classified into Microcapsules with a single core surrounded by a layer of encapsulating material or Microspheres with the core dispersed in a continuous matrix network and more complex structures such as multilayer microcapsules or multi-shell microspheres. Microparticles can be made from either synthetic polymers or natural polymers or mixed systems, depending on the intended application (reviewed by Chen et al., 2006). Examples of microparticles include those made from synthetic polymers such as poly (lactic acid) (PLA) or poly-lactic-*co*-glycolic acid (PLGA) (Freitas et al., 2005) and natural polymers such as protein-based, carbohydrate-based, lipid-based or mixed systems as reviewed by the following: Augustin and Hemar (2009); Benshitrit, Levi, Tal, Shimoni and Lesmes (2012).

However, in spite of successful elaboration of many synthetic polymers as delivery systems, these cannot be used in food applications (Peppas et al., 2000), that require materials that are generally recognized as safe (GRAS) substances. Thus among food

biopolymers, food proteins are being widely investigated for formulation of microparticles for food applications (reviewed by Sinha and Trehan, 2003).

2.1.2 Protein microparticles

Food proteins are a versatile group of biopolymers that carry an important nutritional value along with considerable functionality (reviewed by Benshitrit et al., 2012). Therefore, interest in developing protein microparticles as delivery systems has grown. Various kinds of animal proteins including gelatin (Strauss and Gibson, 2004), collagen (reviewed by Gómez-Guillén, Giménez, López-Caballero, Montero, 2011), casein (reviewed by Elzoghby, Samy and Elgindy, 2012), albumin (MacAdam, Shaft, James, Marriott and Martin, 1997) and whey protein (Gunasekaran, Ko, Xiao, 2007) have been investigated including plant proteins such as soy, pea, rice (reviewed by Nesterenko et al., 2013), maize zein (Liu, Sun, Wang, Zhang, and Wang, 2005), wheat gliadin (reviewed by Chen et al., 2006) and sorghum kafirin (Taylor et al., 2009a).

Proteins have several useful attributes as reviewed by Nesterenko et al. (2013), which include: biocompatibility, biodegradability, good amphiphilic and functional properties such as water solubility, and emulsifying and foaming capacity. The use of plant proteins as wall foaming materials in microencapsulation reflects the present ‘green’ trend in the pharmaceutical, cosmetics and food industry. In food applications, plant proteins are known to be less allergenic compared to animal-derived proteins (Jenkins et al., 2007).

According to Augustin and Hemar (2009), the properties of proteins are influenced by their amino acid composition, conformation and charge as well as their denaturation temperature. Because of their amphiphilic nature, proteins can self-assemble. Thus, this aggregation of protein enables the formation of microparticles.

2.1.3 Formation of microparticles

Protein microparticles can be formed by self-association/assembly or controlled aggregation (reviewed by Matalanis et al., 2011). Whitesides, Mathias and Seto (1991) define molecular self-assembly as the spontaneous organization of molecules under thermodynamic equilibrium conditions into structurally well-defined and rather stable

arrangements (aggregates) through a number of non-covalent interactions. These non-covalent interactions include hydrogen bonds, ionic bonds and van der Waals' forces which are all collectively responsible for the assembly of protein molecules into some well-defined and stable structures (Zhang, 2002). Also, when proteins are heated above their thermal denaturation temperature, protein self-association is promoted through hydrophobic attraction and disulphide bond formation. When a protein is denatured, its physical and chemical interactions change appreciably through exposure of non-polar amino acids for example leucine or valine and sulphur-containing groups such as cysteine, originally present within the compact interior of the protein (reviewed by Augustin and Hemar, 2009; Jones and McClements, 2010; Matalanis et al., 2011). The nature of the particles formed can be controlled by manipulating the intermolecular interactions, through controlling pH, ionic strength and heating conditions (reviewed by Matalanis et al., 2011). Consequently, denatured proteins have a greater tendency to aggregate with each other through hydrophobic bonding and disulphide bond formation (reviewed by Jones and McClements, 2010). Protein aggregation is defined as a universal term for the summary of protein species formed by covalent bonds or non-covalent interactions (Mahler, Friess, Grauschopf and Kiese, 2009).

Protein aggregation can be induced by a wide variety of conditions, including temperature, mechanical stress such as shaking and stirring, pumping, freezing and/or thawing and formulation parameters such as protein concentration, pH, salt concentration, salt type and solvent added (Van der Linden and Venema, 2007; Mahler et al., 2009). In food applications, aggregation is often triggered by acidification, which neutralises the charge on the protein as the pH approaches the isoelectric point (pI) of the protein, or by heating which causes unfolding and exposure of hydrophobic groups (reviewed by Augustin and Hemar, 2009).

The aggregation process may lead to soluble and/or insoluble aggregates, which may precipitate (Mahler et al., 2009). The morphology of the insoluble aggregates may be in the form of amorphous (also known as non-linear, aggregate) or fibrillar (also referred to as linear) material (reviewed by Chen et al., 2006; Van der Linden and Venema, 2007; Mahler et al., 2009). The fibrillar form is created by linear aggregation of structural units maintained by hydrophobic interactions, whereas the aggregate form is produced by

random aggregation of structural units essentially controlled by Van der Waals forces (reviewed by Chen et al., 2006).

According to reviews by Jones and McClements (2010) and Chen et al. (2006), linear morphologies are formed when there is relatively strong repulsion between the protein molecules, low ionic strength and also when proteins are heated far from their isoelectric point. Large spheroid particulate aggregates tend to be formed under conditions where there is only a weak electrostatic repulsion between the protein molecules or high ionic strength or pH adjusted close to the proteins isoelectric point. As aggregation is not specific (Mahler et al., 2009), the formation of a three-dimensional network varies (reviewed by Augustin and Hemar, 2009). To illustrate the structural form that arises from the assembly of proteins, Van der Linden and Venema (2007) using β-lactoglobulin as a model protein showed how the structure of the protein built from heating at 80°C varies with the pH of the solution (Figure 1).

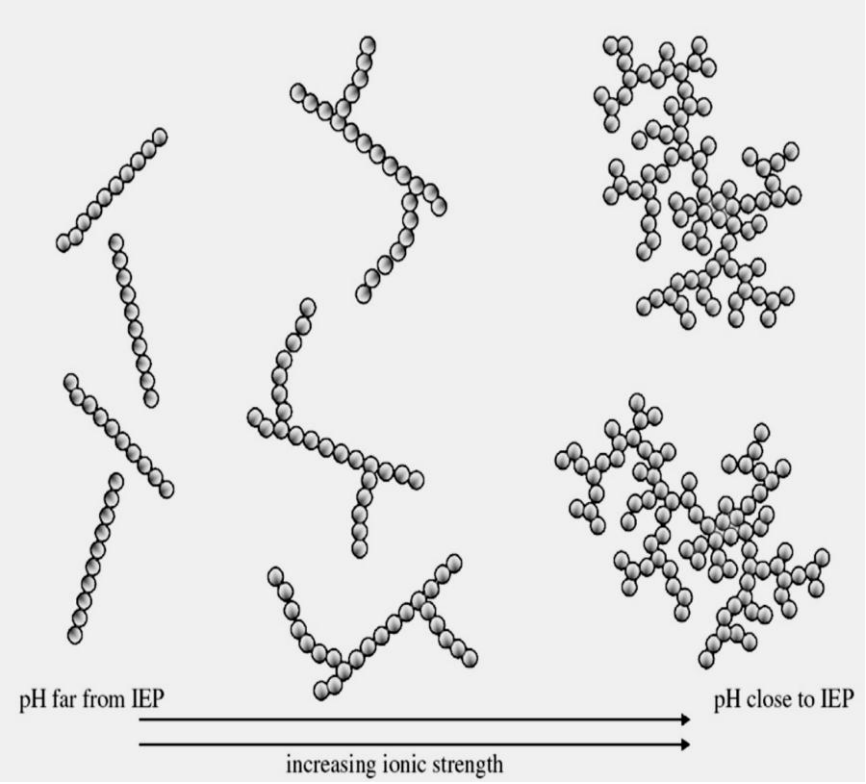


Figure 1: Schematic representation of protein structures formed by heat-denatured globular protein solutions as a function of the ionic strength and difference between the IEP or pI of the protein and the pH of the solution (Van der Linden and Venema, 2007)

For a pH near the isoelectric point (pI or IEP) of the protein (where the overall charge of the protein is zero), spherical aggregates are formed, while for a pH far from the pI, linear aggregates are formed.

According to Mahler et al. (2009), the protein aggregates can be categorized based on different aspects such as the type of bond, reversibility and size of the aggregates. Non-covalent aggregates are formed solely via weak forces such as van der Waals forces, hydrogen bonding, hydrophobic and electrostatic interactions, whereas covalent aggregates may, for example form via disulphide bond linkages (reviewed by Jones and McClements, 2010). Aggregation may be reversible or irreversible, where the irreversible aggregates could be permanently eliminated by preparative separation processes such as filtration techniques (Van der Linden and Venema, 2007). The formation of reversible aggregates is often considered to be caused by the self-assembly of protein molecules, which can be induced by changes in pH or ionic strength of the protein solution (Gerrard, 2002; reviewed by Jones and McClements, 2010).

Therefore, understanding the aggregation process of proteins may be useful in fabrication of protein microparticles for various applications. For example, casein microparticles can be formed by controlled aggregation by adjusting the pH close to the proteins pI, by adding multivalent counter ions or by adding chymosin (Copper, Corredig and Alexander, 2010).

2.1.4 Processes for preparing protein microparticles

Most methods used are modifications of three basic techniques: Spray drying, Solvent extraction/evaporation and Coacervation (phase separation) (Chen and Subirade, 2007). As reviewed by Sinha and Trehan (2003), the choice of method depends not only on the properties of the protein but also on the intended microparticle use (Herrmann and Bodmeier, 1998).

2.1.4.1 Spray drying

Spray drying is a technique wherein atomized protein suspensions or solutions are quickly dried using a heated gas (reviewed by Jones and McClements, 2010). Depending on the starting feed protein solution and operating conditions, a very fine powder (10-50 µm) or

large size particles (2-3 mm) are produced (reviewed by Gharsallaoui, Roudaut, Chambin, Voilley and Saurel, 2007; reviewed by Murugesan and Orsat, 2011). Spray-drying is relatively simple, fast, cheap and of high throughput but cannot be used for highly temperature-sensitive compounds. Moreover, control of particle size is difficult, and yields for small batches are moderate (Freitas et al., 2005).

2.1.4.2 Solvent extraction/evaporation

Microparticle preparation by solvent extraction/evaporation basically consists of four major steps: (i) dissolution or dispersion of the protein in an organic solvent, (ii) emulsification of this organic phase in a second phase immiscible with the first one (iii) extraction of the solvent from the dispersed phase by continuous phase, often accompanied by solvent evaporation or extraction resulting in the formation of microparticles and (iv) harvesting and drying of the microparticles. The solvent extraction method does not require elevated temperatures nor phase separation inducing agents (Sinha and Trehan, 2003; Freitas et al., 2005).

2.1.4.3 Coacervation (phase separation)

The coacervation process is a modified emulsification technology (De Vos, Faas, Spasojevic and Sikkema, 2010) and is also known as phase separation (Wilson and Shah, 2007). It is often regarded as the original method for encapsulation (Risch, 1995). As reviewed by Nesterenko et al. (2013), coacervation is carried out by precipitation of biopolymer around the active core under the effects of: change of pH or temperature, addition of a non-solvent or electrolyte compound. During coacervation, a protein is dissolved in a solvent, which is normally aqueous, while continuing to stir the solution, an anti-solvent normally non-miscible solvent is slowly added to the solution (reviewed by Sinha and Trehan, 2003). As a result, there is separation from solution of colloid particles which then agglomerate into separate phases (Korus, 2001).

When a solution of proteins of opposite charge is mixed, a complex is formed. Many factors including the protein type (molar mass, flexibility and charge), pH, ionic strength, concentration and the ratio of the biopolymers affect the strength of the interaction between the biopolymers and the nature of the complex formed in coacervation (reviewed by Jones

and McClements, 2010). Although electrostatic interactions are considered to drive the interaction between biopolymers of opposite charge, hydrophobic interactions and hydrogen bonding can also contribute significantly to the complex formation. Depending on the conditions and the polymers involved, coacervation can be classified as simple or complex. Simple coacervation involves only one type of biopolymer whereas complex coacervation involves the use of two oppositely charged biopolymers (Burgess and Hickey, 1994; reviewed by Augustin and Hemar, 2009).

2.1.5 Application of protein microparticles

Protein microparticles are widely used in microencapsulation (reviewed by Chen et al., 2006). Desai and Park (2005) defines microencapsulation as the technology of packaging solid, liquid and gaseous materials in small capsules or microparticles that release their contents at controlled rates over prolonged periods of time. According to Shahidi and Han (1993), the main objective of encapsulation is to protect the core material from adverse environmental conditions, such as undesirable effects of light, moisture and oxygen and to promote controlled liberation of the encapsulate. However, in food applications, several purposes are targeted as summarized by Desai and Park (2005): (i) protection of the core material from degradation by reducing its reactivity to its outside environment; (ii) reduction of the evaporation or transfer rate of the core material to the outside environment; (iii) modification of the physical characteristics of the original material to allow easier handling; (iv) tailoring the release of the core material slowly over time, or at a particular time; (v) to mask unpleasant flavour or taste of the core material; (vi) dilution of the core material when only small amounts are required, while achieving uniform dispersion in the host material and (vii) to help separate the components of the mixture that would otherwise react with one another.

Protein-based microparticles have found wide and rapidly increasing applications in the food industry because they can be precisely designed for use in many food formulations and virtually any ingredient can be encapsulated, whether hydrophobic, hydrophilic, or even microbial (reviewed by Chen et al., 2006). Incorporating drugs, unsaturated fatty acids, vitamins, probiotics as well as bioactive peptides into animal protein-based microparticles (gelatine, whey, casein, collagen, albumin and elastin) has been done (reviewed by Chen et al., 2006; Elzoghby et al., 2012). Beaulieu, Savoie, Paquin and

Subirade (2002), using whey protein microparticles were able to encapsulate vitamin B with good release properties on digestion. Recent work on encapsulation of β-carotene has been carried out by Cornacchia and Roos (2011) in whey/ sodium caseinate -stabilized oil-in-water delivery system.

2.2 PROTEIN MICROPARTICLES AS DELIVERY VEHICLES FOR NUTRACEUTICALS

The recent advances in food and nutritional science support the concept that diet plays a significant role in improved state of well-being, prevention and mitigation of certain diseases and important part of a healthy lifestyle. This has been due to the realisation that incorporation of certain food products like fruits, cereals and vegetables in the diet can lead to preservation of good, long-term health (Waxman and Norum, 2004). The long term physiological benefit of these foods has been linked to certain bioactive compounds known as nutraceuticals. Health Canada (1998) defines nutraceuticals as a product isolated or purified from foods that is generally sold in medicinal forms not usually associated with food, with physiological benefits or protection aganist chronic disease. Examples of nutraceuticals include probiotics and prebiotics, vitamins, phenolics, phytosterols and certain polyunsaturated fatty acids (Kalia, 2005). Therefore, according to Chen and Subirade (2009) interest in delivery systems for these nutraceutical products has been increasing due to the growing evidence that they confer health benefits. In this review section, specific attention will be given to phenolics as a nutraceutical that requires to be microencapsulated.

2.2.1 Phenolics

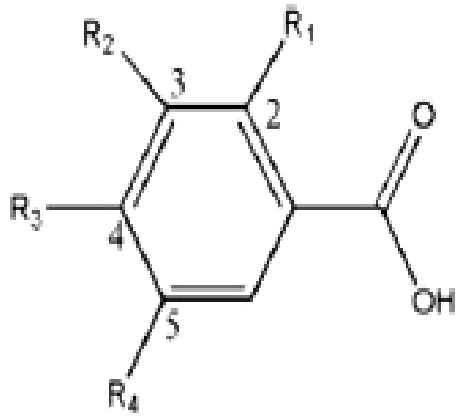
Phenolics form a large group of phytochemicals, which are produced by plants as secondary metabolites to protect them from photosynthetic stress and reactive oxygen species (reviewed by Das, Bhaumik, Raychaudhuri and Chakraborty, 2011). Phenolics, generally, polyphenolic compounds are approximately 8000 naturally occurring compounds, all of which possess one common structural feature, a phenol (an aromatic ring bearing at least one hydroxyl substituent) (Robbins, 2003). They occur primarily in the conjugated form, with one or more sugar residues linked to the hydroxyl groups, although direct linkages of the sugar unit to an aromatic carbon atom also exist. The associated

sugars can be present as monosaccharides, disaccharides or even oligosaccharides (Robbins, 2003).

The main classes of phenolics are defined according to the nature of their carbon skeleton: phenolic acids, flavonoids and the less common stilbenes and lignans (Prakash and Gupta, 2009). All plant-based foods have phenolics (reviewed by Naczk and Shahidi, 2004), and for the purpose of this review, more attention will be given to sorghum as a rich source of phenolics. All sorghums contain phenolics (Dykes, Rooney, Waniska and Rooney, 2005) and according to Hahn, Rooney and Earp (1984), three main classes of phenolics in sorghum can occur, namely phenolic acids, flavonoid-type compounds and condensed tannins.

2.2.1.1 Phenolic acids

The phenolic acids in sorghum largely exist as benzoic or cinnamic acid derivatives (Figure 2), to form hydrobenzoic or hydrocinnamic acids, respectively (reviewed by Awika and Rooney, 2004). The benzoic acid derivatives include gallic, p-hydrobenzoic, vanillic, syringic and protocatechuic acids (Figure 2) and have a C6-C1 structure (reviewed by Dykes and Rooney, 2006). The cinnamic acid derivatives have a C6-C3 structure and include coumaric, caffeic, ferulic and sinapic acids (Figure 2). According to Hahn et al. (1984), the phenolic acids in sorghum are present mostly in bound form with ferulic acid being dominant. Free phenolic acids are found in the outer layers of the kernel (pericarp, testa and aleurone), whereas the bound phenolic acids are associated with the cell walls.



Benzoic acids (**11-16**)

Gallic acid (**11**): $R_1 = H, R_2 = R_3 = R_4 = OH$

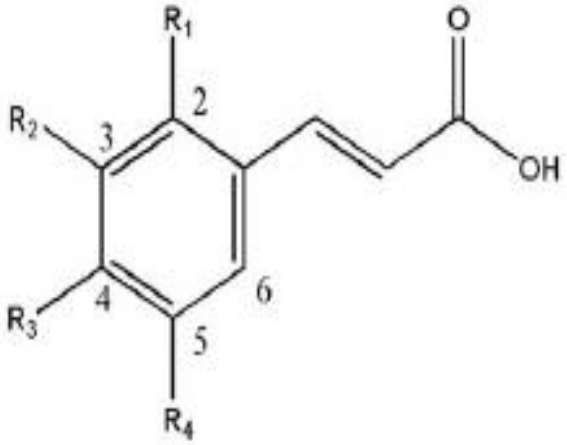
Gentisic acid (**12**): $R_1 = R_4 = OH, R_2 = R_3 = H$

Salicylic acid (**13**): $R_1 = OH, R_2 = R_3 = R_4 = H$

p-hydroxybenzoic acid (**14**): $R_1 = R_2 = R_4 = H, R_3 = OH$

Syringic (**15**): $R_1 = H, R_2 = R_4 = OCH_3, R_3 = OH$

Protocatechuic(**16**): $R_1 = R_4 = H, R_2 = R_3 = OH$



Cinnamic acids (**17-21**)

Cinnamic acids (**17-21**)

Caffeic acid (**17**): $R_1 = R_4 = H, R_2 = R_3 = OH$

Ferulic acid (**18**): $R_1 = R_4 = H, R_2 = OCH_3, R_3 = OH$

o-coumaric acid (**19**): $R_1 = OH, R_2 = R_3 = R_4 = H$

p-coumaric acid (**20**): $R_1 = R_2 = R_4 = H, R_3 = OH$

Sinapic (**21**): $R_1 = H, R_2 = R_4 = OCH_3, R_3 = OH$

Figure 2: Basic structure of phenolic acids found in sorghum grain (Awika and Rooney, 2004)

2.2.1.2 Flavonoids

As reviewed by Tripoli, Guardia, Giammanco, Majo and Giammanco` (2007), the basic structure of flavonoids, consists of a fused A and C ring, with the phenyl ring B attached through its 1` position to the 2-position of the C ring (represented by the labelling on flavonol structure in Figure 3 below). Flavonoids can be divided into several classes according to the degree of oxidation of the oxygen heterocycle: flavones, flavonols, isoflavones, anthocyanins, flavanols and flavanones (Figure 3). Sorghum, amongst all cereals has the widest variety of flavonoids (reviewed by Dykes and Rooney, 2006) of which the anthocyanins are the major class of flavonoids in pigmented sorghum types.

The term anthocyanin refers to the glycosides of anthocyanidin (for example pelargonidin, malvidin, cyanidin) (reviewed by Bravo, 1998). Sorghum anthocyanins are unique in that some do not contain the hydroxyl group in the C-3 position and thus they are called 3-deoxyanthocyanins (reviewed by Dykes and Rooney, 2006). Further, they are reported to be more stable in acidic solution relative to the anthocyanidins commonly found in most food plants (Awika, Rooney and Waniska, 2004a) due to the lack of oxygen at C-3 believed to improve their stability. The two most common 3-deoxyanthocyanins in sorghum are apigeninidin and luteolinidin, which are responsible for the yellow and red colours, respectively (Awika et al., 2004a; Awika, Rooney and Waniska, 2004b). Dykes and Rooney (2007) also reported other 3-deoxyanthocyanins in sorghum which were apigeninidin-5-glucoside, 5-methoxyapigeninidin among others. Other flavonoids isolated and identified in sorghum grains include the flavones apigenin and luteolin, which are predominant in tan- plant sorghums (Awika et al., 2004a) and flavonones eriodictyol, naringenin and eriodictyol glucoside (Dykes, Seitz, Rooney and Rooney, 2009).

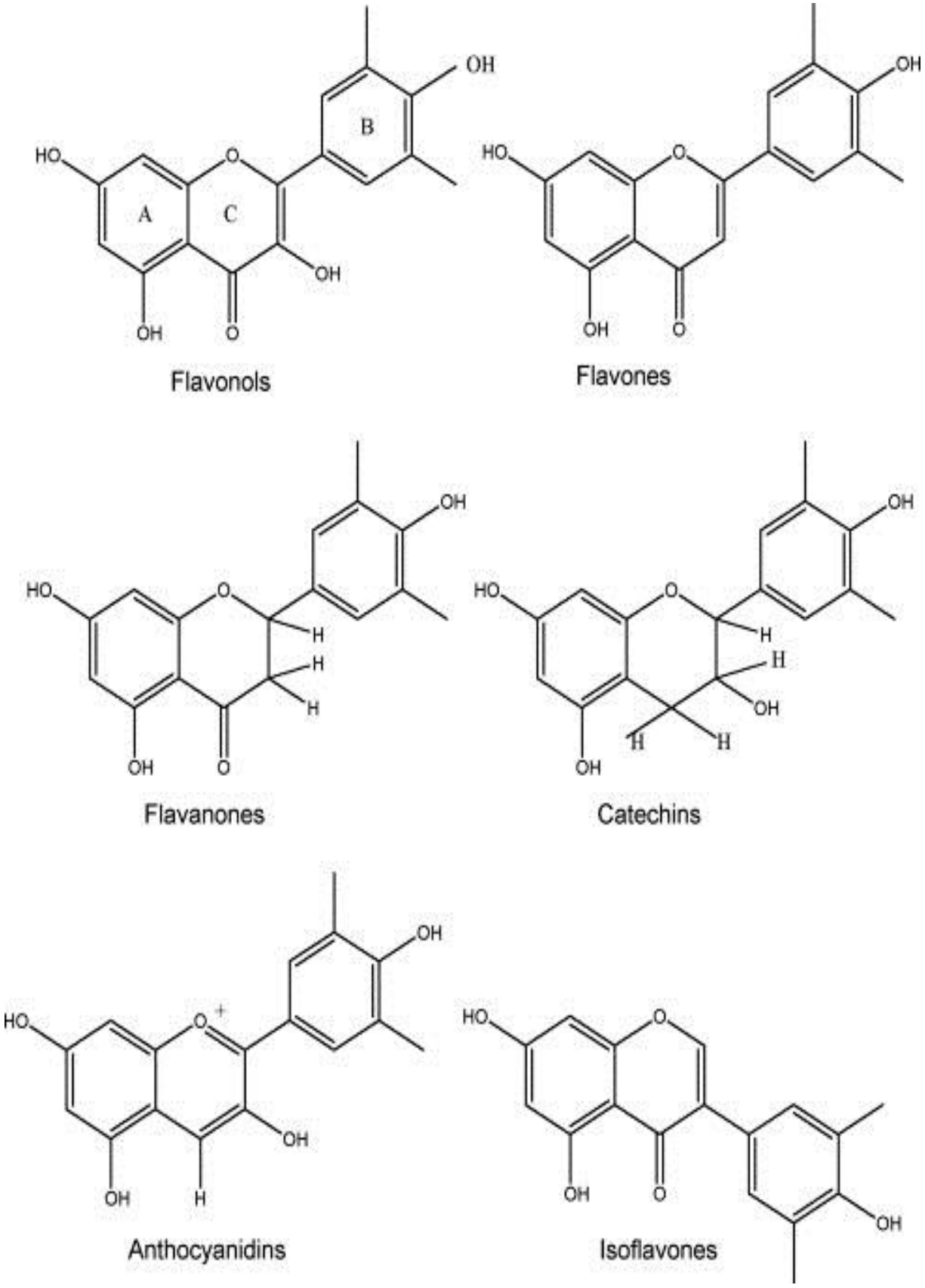


Figure 3: Molecular structures of flavonoids (Tripoli et al., 2007)

2.2.1.3 Tannins

As reviewed by Bravo (1998), tannins are highly hydroxylated molecules that can form insoluble complexes with carbohydrates and proteins. The tannins in sorghum are of the condensed type. Condensed tannins consist of polymerized flavanol units (Figure 4) (Dykes and Rooney, 2007) and occur in sorghum types with a pigmented testa layer (Dykes and Rooney, 2007). According to Butler (1982) and Dykes and Rooney (2007), the condensed tannins in sorghum often exist as oligomers of five to seven flavan-3-ols (Figure 4) which depolymerise into monomeric anthocyanidin pigments and thus are designated as proanthocyanidins. The autooxidative or enzymatic polymerization of flavan-3-ol and flavan-3, 4-diol units, linked by C4 → C8 interflavan bonds (also known as the B-type linkage) (Figure 4) has been suggested as the process leading to the formation of high-molecular weight condensed tannins (reviewed by Bravo, 1998).

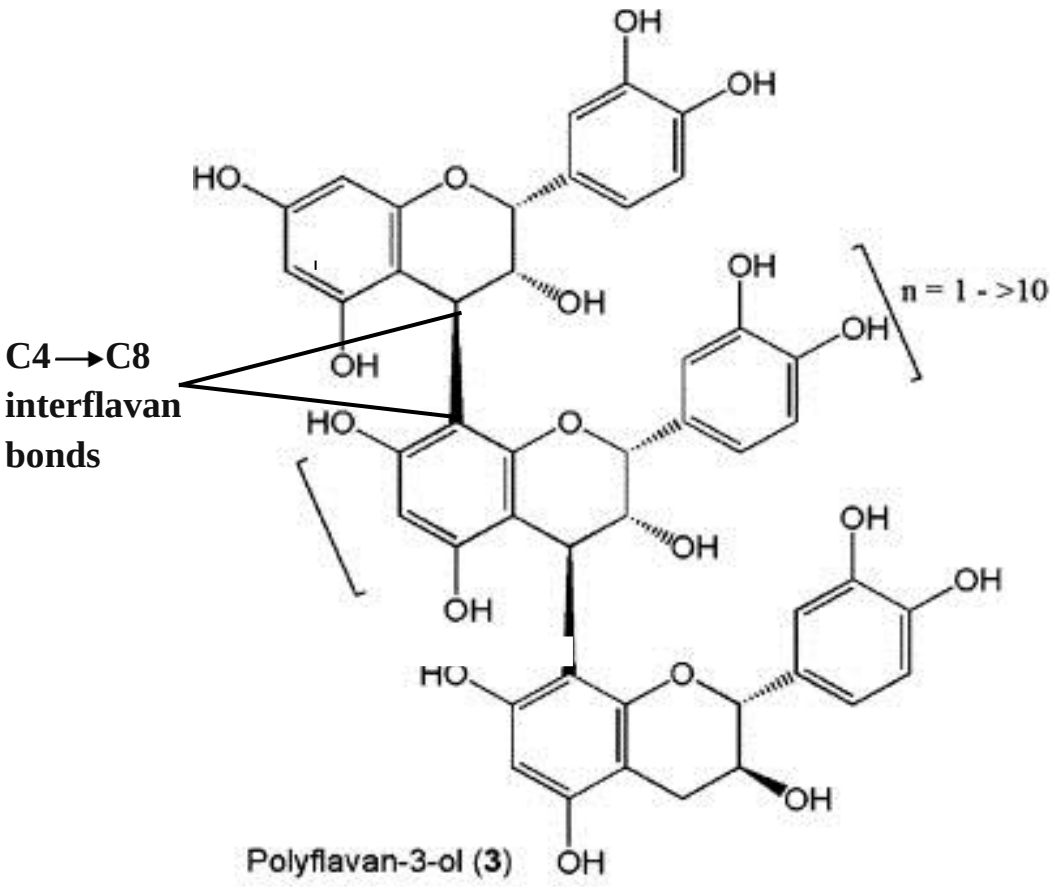


Figure 4: Structure of proanthocyanidin (condensed tannin) found in sorghum, a polyflavan-3-ol with a B-type linkage (Dykes and Rooney, 2007)

2.2.2 Antioxidant and health-promoting activity of phenolics

As reviewed by Wong, Leong and Koh (2006), free radicals, in the form of reactive oxygen and nitrogen species, are an integral part of normal physiology. An over-production of these reactive species or free radicals causes an imbalance in the body's antioxidant defence system and this leads to oxidative stress. These reactive species can react with biomolecules, causing cellular injury and death which may lead to the development of chronic diseases such as cancers and those that involve the cardio- and cerebrovascular systems. However, dietary antioxidants can augment cellular defences and help to prevent oxidative damage to cellular components (Conklin, 2000; Prakash and Gupta, 2009; Das et al., 2011).

Phenolics are antioxidants, due to the reactivity of the phenol moiety (hydroxyl substituent on the aromatic ring (Robbins, 2003). Substituents on the aromatic ring affect the stabilization and radical-quenching ability of these phenolics. Therefore different phenolics exhibit different antioxidant activity (reviewed by Rice-Evans, Miller and Paganga, 1997). Although there are several mechanisms, the predominant mode of antioxidant activity is believed to be radical scavenging via hydrogen atom donation (Robbins, 2003). Other established antioxidant, radical quenching mechanisms are through electron donation and singlet oxygen quenching (reviewed by Rice-Evans et al., 1997). According to a review by Bravo (1998), phenolics function as terminators of free radicals and chelators of metal ions that are capable of catalyzing lipid peroxidation. They do so by interfering with the oxidation of lipids and other molecules by rapid donation of a hydrogen atom to radicals.

Through epidemiological studies, convincing evidence has been shown that diet rich in dietary antioxidants is associated with a lower incidence of degenerative diseases (Prakash and Gupta, 2009). Also, substantial evidence *in vitro* has suggested that phenolics can affect numerous cellular processes like, gene expression, apoptosis, platelet aggregation, intercellular signalling, that can have anti-carcinogenic implications (reviewed by Das et al., 2011). These apart, phenolics have been shown to possess antioxidant, anti-inflammatory, anti-microbial, cardio protective activities and play a role in the prevention of neurodegenerative diseases and diabetes mellitus (Scalbert, Johnson and Saltmarsh, 2005a). It is well established that some phenolics, administered as supplements or with food, do improve health status, as indicated by several biomarkers closely associated with

cardiovascular risk (Scalbert, Manach, Morand, Rèmèsy and Jimènez, 2005b). In contrast, evidence for protective effects of phenolics against cancers, neurodegenerative diseases, and brain function deterioration is still largely derived from animal experiments and *in vitro* studies (Lambert, Hong, Yang, Liao and Yang, 2005).

Concerning flavonoids, luteolin has been shown to be powerful antioxidant that inhibits the oxidation of low-density lipoprotein (LDL) *in vitro*, a major factor in the promotion of atherosclerosis, which is a plaque build-up in arteries that can lead to heart attack or stroke (Marchand, 2002). As reviewed by Bravo (1998), flavonoids are the most effective scavengers of free radicals, responsible for DNA damage and tumour promotion. Their anticarcinogenic activity is expressed by multiple mechanisms, like activating and enhancing activities of antioxidant enzymes or by inhibiting certain enzymes such as P-450 found in the liver (Prakash and Gupta, 2009).

However, as reviewed by Chen et al. (2006), the effectiveness of nutraceutical products such as phenolics, in preventing diseases depends on preserving their bioavailability. This represents a formidable challenge, given that only a small proportion of molecules remain available following oral administration, due to insufficient gastric residence time, low permeability and/or solubility within the gut, as well as instability under conditions encountered in food processing (temperature, oxygen, light) or in the gastrointestinal (GI) tract (pH, enzymes, presence of other nutrients), all of which limit the activity and potential health benefits of nutraceutical molecules Thus, the delivery of these molecules requires protective mechanisms that: (1) maintain the active molecular form until the time of consumption and (2) deliver this form to the physiological target within the organism. Hence, microencapsulation technology is a promising approach to producing stable, viable systems (Fang and Bhandari, 2010).

2. 3 FUNCTIONAL PROPERTIES OF PROTEIN MICROPARTICLES

The functional characteristics of protein microparticles depend on their structure. Particle size and shape is very important because it impacts both the physicochemical properties and sensory attributes of food (reviewed by Jones and McClements, 2010; Benshitrit et al., 2012). The particle size of the protein microparticle is also significant as it determines the encapsulation rate, the rate of release of encapsulated compound and its specific

application (Freitas et al., 2005). According to a review by Chen et al. (2006), larger microparticles generally release encapsulated compounds more slowly and over longer time periods, while particle size reduction introduces several bio-adhesive improvement factors, including increase adhesive force and prolonged GI transit time, leading to a higher bioavailability of the compound. For example, zein microparticles for delivery of ivermectin with an average diameter of 1 µm were found suitable for phagocytosis by macrophages (Liu, Sun, Wang, Zhang and Wang, 2005). The small size of the zein microparticles, lead to faster release of encapsulated compound, because of their increased ratio to volume. According to Dubey et al. (2009), the total surface area of the microparticle is inversely proportional to its size.

As reviewed by Chen et al. (2006); Jones and McClements (2010), protein microparticles with different shapes can often be formed, for example spheres, spheroids, rods or clusters. Mostly spheres are produced by application of shear forces or extrusion methods during particle formation (Norton and Frith, 2001). The appearance, rheology and release characteristics of colloidal dispersions containing non-spherical particles are often different from those containing a similar amount of spherical ones. Consequently, modulations of particle shape can be used to create novel or improved textures in food (reviewed by Jones and McClements, 2010).

Lastly, the internal structure of the protein microparticle is crucial in determining its functional characteristics such as encapsulation efficiency, loading capacity, permeability, integrity and digestibility. In particular, particle porosity has a major influence on the accessibly of a bioactive trapped within its matrix. In general, a highly porous structure should allow for relatively easy access and release of an encapsulated bioactive while a dense structure would limit access and release (reviewed by Matalanis et al., 2011).

2.3.1 Plant-based protein microparticles

Despite the possible advantage of absorbability and low toxicity of degradable end products, most animal-based protein microparticles (hydrophilic-based systems) has shown a number of drawbacks (Vandelli, Rivasi, Guerra, Forni and Arletti, 2001). According to reviews by Elzoghby et al. (2012) and Nesterenko et al. (2013), these drawbacks include difficulties in achieving sustained ingredient release because of their rapid solubilisation in

aqueous environments, thus resulting in fast ingredient release profiles. When the system absorbs water and swells, encapsulated materials will rapidly diffuse out (reviewed by Elzoghby et al., 2012).

However, in order to overcome this problem, chemical cross-linking procedures (e.g. glutaraldehyde and formaldehyde treatment) has been examined (Hennink and van Nostrum, 2012) reducing protein dissolution and drug release at body temperature by the formation of non-soluble networks on the microparticle surface (Esposito, Cortesi and Nastruzzi, 1996). Unfortunately, the presence of residual cross-linking agents could lead to toxic side effects, in addition, unwanted reactions between the drug and cross-linker that could result in the formation of toxic or inactivated derivatives (reviewed by Vandelli, Romagnoli, Monti, Gozzi, Guerra, Rivasi and Forni, 2004).

In contrast to using hydrophilic animal-based protein microparticles, hydrophobic plant proteins such as zein (Liu et al., 2005; reviewed by Reddy and Yang, 2011), gliadin (reviewed by Elzoghby et al., 2012) and kafirin (Taylor et al., 2009b) have the capability of yielding sustained ingredient release. Moreover, plant proteins reduce the risk of spreading diseases such as bovine spongiform encephalitis (mad cow disease) (Lai and Guo, 2011). As reviewed by Reddy and Yang (2011), plant proteins are more widely available, biodegradable and have considerably less potential to be immunogenic compared with animal protein such as bovine collagen. More attention will be drawn to kafirin as a hydrophobic plant protein (reviewed by Duodu, Taylor, Belton and Hamaker, 2003) of potential value for preparation of microparticles (Taylor et al., 2009a).

2.3.2 Kafirin

Kafirins are the sorghum prolamin storage proteins, which account for up to 82 % of the protein in the endosperm (reviewed by Belton, Delgadillo, Halford and Shewry, 2006). Kafirins are rich in glutamine, proline, leucine and proline, with very small amounts of lysine (Taylor and Belton, 2002). Mazhar, Chandrashekar and Shetty (1993); Shull, Watterson and Kirleis (1991), identified and divided kafirins into 3 major subclasses, namely α-, β- and γ-kafirin, based on their relationships to the zeins (maize protein) as revealed by their amino acid compositions and sequences, molecular masses and their immunochemical cross-reactions. A fourth group, δ-kafirin related to the δ-zeins of maize,

has been identified (reviewed by Belton et al., 2006). According to Watterson, Shull and Kirleis (1993), sorghum endosperm, whether floury or corneous, consists of some 66-84 % α-kafirin, 7-8 % β-kafirin in the corneous tissue; 10-13 % in the opaque endosperm and 19-22 % γ-kafirin in the floury endosperm; 1-12 % in the corneous endosperm. The α-kafirin resolves into two bands, resolved by SDS-PAGE of about 25,000 (α_1-kafirin, equivalent to Z22), and 23,000 (α_2-kafirin, equivalent to Z19), (Shull et al., 1991; El Nour, Peruffo and Curioni, 1998). As reviewed by Duodu et al. (2003), α_1-kafirin has two cysteine residues and α_2-kafirin has only one cysteine residue, resulting in different functionalities.

On the other hand, β-kafirin has ten cysteine residues and appears mostly as oligomers and polymers. El Nour et al. (1998) hypothesised that β-kafirin (which like γ-kafirin is rich in cysteine) could act as a chain extender by linking together oligomers of γ- and α_1-kafirin by disulphide bridges to form high molecular weight polymers. On the other hand, the α2-kafirin (lower in cysteine) could act as a chain terminator by preventing the possibility of bonding with β-kafirin and formation of high molecular weight polymers. Gamma-kafirin appears as polymers stabilized by disulphide bonds (Belton et al., 2006). Gamma-kafirin is the most proline rich of the kafirin subclasses and contains the most proline repeats (reviewed by Shewry, 2002). Table 1 shows the comparison of the properties of different kafirin subclasses in total kafirin.

Table 1: Comparison of the properties of the four subclasses of kafirin

	Molecular mass	**No. of amino acid residues**	**Amino acid composition**	**Polymerisation behaviour**	**No. of genes**
α-	26,000-27,000	240-250	Rich in non-polar amino acids, no Lys, one Trp, 10 blocks of repeated amino acids	Monomers, oligomers and polymers	Approx. 20
β-	18,745	172	Rich in Met and Cys, two Trp	Monomers and polymers	1
γ-	20,278	193	Rich in Pro, Cys, His, No Lys, Asn, Asp, Trp. Four repeats (consensus Pro, Pro, Pro, Val, His, Lys)	Oligomers and polymers	1/2
δ-	12,961	114	Rich in Met, no Lys, 1 Trp	Not known	Not known

Adapted from Belton et al. (2006)

The secondary structure of kafirin (since no models exist) is believed to be an α-helix conformation (Gao, Taylor, Wellner, Byaruhanga, Parker, Mills and Belton, 2005). This is due to the kafirin similarity to zein (DeRose, Ma, Kwon, Hasnain, Klassy and Hall, 1989), thus the same model used for zein can also apply to kafirin. Two functional properties of kafirin which are influenced by its amino acid composition and secondary structure are hydrophobicity and protein digestibility, both of which are expected to influence the properties of kafirin microparticles (Taylor, 2008).

2.3.2.1 Kafirin Microparticles

Kafirin microparticles (KEMs) can be formed through phase separation by an organic acid such as glacial acetic acid (Taylor et al., 2009a). The KEMs were spherical or irregular in shape with many internal vacuoles. As stated, KEMs have potential to be used as controlled release delivery devices of encapsulated polyphenols (Taylor et al., 2009b) or as natural non-animal protein bioactive scaffolds (Anyango, Duneas, Taylor and Taylor, 2012).

2.4 ALTERATION OF THE FUNCTIONAL PROPERTIES OF PROTEIN MICROPARTICLES

In many situations, the initial protein microparticles formed are not physically stable and are highly prone to coalescence and phase separation (reviewed by Jones and McClements, 2010). Also the protein microparticle size may be too small or big for a specific application (Freitas et al., 2005; reviewed by Matalanis et al., 2011). In addition, some plant-based protein microparticles have inferior mechanical properties, especially poor hydrolytic stability (reviewed by Reddy and Yang, 2011). Consequently, it becomes necessary to cross link the protein within the particles to increase the stability and size of the protein microparticles. According to Peppas et al. (2000) and Hoffman (2002), cross linking improves the thermal and mechanical stability of protein microparticles and can be tailored to modify the release rate of the incorporated active ingredient in terms of their morphology. Cross-linking can be carried out using various physical and chemical methods depending on the specific characteristics of the proteins involved.

2.4.1 Physical cross-linking

Physical cross-linking refers to any process that adds stability to the protein microparticles through the formation of non-covalent bonds (Hennink and van Nostrum, 2012), such as hydrogen bonding, hydrophobic association and ion bridging (reviewed by Jones and McClements, 2010).

2.4.1.1 Temperature

Use of heat (thermal denaturation) has been investigated to cross link proteins, for example albumin (Patil, 2003), gelatin (Vandelli et al., 2004) and whey (Zhang and Zhong, 2009). Temperatures above 50^{o}C during preparation of albumin microparticles resulted in insoluble microparticles due to formation of inter-chain amide links (Patil, 2003). Picot and Lacroix (2004) mixed bifidobacteria cultures with heat treated pre-denatured whey-protein solutions at 40^{o}C and subsequently spray dried these mixtures to achieve water-insoluble microparticles. This allowed for the sustained delivery of the viable bacteria in stable protein microparticles. Anyango et al. (2012) cross-linked already formed kafirin microparticles by wet heat treatment. An increase in overall size of the KEMs from roughly 5 µm to 20 µm was observed with an increase in the vacuole size. These authors attributed the increased vacuole size with heat treatment to greater expansion of air within the microparticles since the vacuoles were probably footprints of air bubbles (Taylor et al., 2009a). The cross linking by heat treatment was due to kafirin polymerization by disulphide bonding following heat treatment (Anyango et al., 2012). Thus the cross-linking procedures of heat treatment lead to an increase size and change of shape with an improvement in the loading capacity of the KEMs (Anyango et al., 2012). The heat treatment was applied to KEMs already formed. Also the use of microwave energy has been investigated in cross linking proteins like gelatin (Vandelli et al., 2004) as in some cases prolonged exposure to heat makes it undesirable to cross-link proteins (reviewed by Chen et al., 2006). Microwave-treated gelatin microparticles at 250^{o}C for 10 min (Vandelli et al., 2004) were shown to be insoluble in water. These authors hypothesized that the cross-linking mechanism was by a condensation reaction between free carboxyl and amino groups of the gelatin protein chain, induced by the drastic removal of water.

2.4.1.2 pH

Addition of mineral ions and changes in pH may be used to promote protein association through alterations in electrostatic interactions (reviewed by Jones and McClements, 2010). According to Jones and McClements (2010), the charge on the proteins can be made to change from positive to negative by adjusting the pH from above to below the proteins pI. Hence, the tendency for aggregation to occur can be controlled by controlling the solution pH. Casein can be gelled by adjusting the pH close to the proteins pI, by adding multivalent counter-ions (Cooper et al., 2010). Lee and Rosenberg (2000) also demonstrated that whey microparticles at pH 5.5 were larger in size and more porous than those prepared at neutral pH.

2.4.1.3 Shear

Shear forces can be used to break up protein microparticles into smaller sizes, in case of higher molecular weight and increased protein concentration which leads to high viscosity of the protein solution (Sinha and Trehan, 2003; reviewed by Jones and McClements, 2010). Also shear forces can be used to cause spherical particles to become increasingly elongated and aggregated (reviewed by Jones and McClements, 2010). Research has shown that small sized microparticles are formed at higher stirring speeds (reviewed by Matalanis et al., 2011) due to stronger comminution of droplets (Betz and Kulozik, 2011). Following the application of shear, the structure of newly formed particles is normally fixed using an appropriate gelation mechanism for example change in temperature or addition of gelling agent (reviewed by Matalanis et al., 2011). Taylor et al. (2009a) demonstrated the effect of shear during the preparation of kafirin microparticles using ethanol as the solvent. These authors attributed the increase in vacuole size due to high turbulence introduced by high shear that allowed for the incorporation of more bubbles into the solution mixture during preparation. These bubbles became entrapped within the microparticles, possibly coalescing to form bigger bubbles as the kafirin precipitated, resulting in bigger vacuole size (imprints of air bubbles). The authors concluded that an alteration on the vacuole size of the kafirin microparticles is possible leading to improved encapsulation capacities.

Betz and Kulozik (2011) also studied the influence of preparation conditions. By increasing the stirrer speed, a reduction in the microcapsule size of whey protein microcapsules was observed due to the stronger comminution of droplets. However, the increased shear rate at 1900 rpm resulted in high droplet collision rates and thus induced aggregation and coalescence of the droplets. As reviewed by Matalanis et al. (2011), there is a limit to this effect as excessively high shear rates can result in protein microparticle aggregation therefore by controlling the shear forces, it is possible to generate protein microparticles with defined size and morphology.

2.4.2 Chemical cross-linking

Chemical cross linking refers to the introduction of a covalent linkage between the protein's functional groups, such as amines, thiols, hydroxyl groups and phenyl rings (reviewed by Jones and McClements, 2010).

2.4.2.1 Aldehyde reactions

Aldehydes such as glutaraldehyde and formaldehyde can be used to chemically cross-link protein microparticles, allowing for stable microcapsule dispersion to be formed plus better mechanical properties (reviewed by Nesterenko et al., 2013). It is well established that the aldehyde group of the glutaraldehyde reacts with the amino group of the lysine residues of the protein chain to from a Schiff base through the establishment of aldimine linkages (CH=N) (Hennink and van Nostrum, 2012). Akin and Hasirci (1995) prepared and characterized gelatin microparticles cross linked with glutaldehyde. These authors found that stable spherical structures of gelatin microparticles (1 µm) could be reproducibly formed by controlling the amount of glutaraldehyde used. When the amount of glutaraldehyde was increased during preparation of the gelatin microparticles, the size of the microparticles decreased, probably due to increased cross-linking density resulting in closely packed structure like helical formation causing shrinkage of the microparticles. Also, they found that increasing cross-linking resulted in slower rate of evaporation leading to the formation of microparticles with a smooth surface.

Damink, Dijkstra, van Luyn, van Wachem, Nieuwenhuis and Feijen (1995) also described collagen microparticles prepared by cross-linking with glutaraldehyde as more stable due

to the formation of covalent cross linkage of collagen fibrils with glutaraldehyde. Whey-based microparticles containing a model drug, theophylline, were prepared using glutaraldehyde-saturated toluene via the organic phase (Lee and Rosenberg, 2000). The process of cross linking was shown to be effective in influencing the rate of core release as water insoluble whey microparticles were formed.

Drug-loaded casein microparticles for sustained release in an oral dosage have been formed via glutaraldehyde cross linking. These cross linked casein microparticles were shown to be resistant to proteolytic enzymes for more than 24 h (Latha and Jayakrishman, 1994). The authors suggested that aldehyde cross linking of casein resulted in the removal of most of the free amino groups in the protein, thus reducing its proteolytic susceptibility and imparting stability to the microparticles in the gastrointestinal tract. Although the use of glutaraldehyde as a cross linker leads to improvement of the mechanical properties and stability of the protein microparticles, importantly its high toxicity may limit the application of the final product (reviewed by Elzoghby et al., 2012).

2.4.2.2 Enzymatic methods

Enzymes can be utilized to catalyze specific cross linking reactions involving protein microparticles in food grade systems. The enzyme, transglutaminase (TGase) has been widely used to cross link various types of proteins in foods (Gerrard, 2002; Nicolai, Britten and Schmitt, 2011; Hennink and van Nostrum, 2012). TGase is a transferase that forms both inter- and intramolecular isopeptide bonds in and between many proteins by cross linking of the amino residues of glutamine and lysine (reviewed by Jones and McClements, 2010). The extent of cross linking can be controlled by changes in pH, enzyme inhibitors or heating (De Jong and Koppelman, 2002). Heidebach, Först and Kulozik (2009) applied a transglutaminase to induce casein microparticle formation by gelation for the microencapsulation of probiotic cells. Covalent cross-linking, as a result of the enzyme reaction, lead to water insoluble and physically stable spherical protein microparticles with average diameters of 165 µm. An encapsulation yield of 70-93% was achieved and the authors showed that the use of enzyme in the process of making casein microparticles for encapsulation of probiotic cells, provided protection for these cells against low pH-values in the human stomach.

2.4.2.3 Natural components

The functional performance of protein microparticles can be improved by using natural compounds. For example, polyphenols are known to react under oxidizing conditions with side chain amino groups of peptides, leading to formation of cross links in proteins (Butler, Riedl, Lebryk and Blytt, 1984; Robbins, 2003; Strauss and Gibson, 2004). Extensive work was done by Strauss and Gibson (2004) who used plant-derived phenolic acids and flavonoids to prepare cross-linked gelatin gels and cross linked gelatine pectin coacervates in the form of microparticles for use as food ingredients. Gels cross-linked by these phenolics had greater mechanical strength, reduced swelling, and fewer free amino groups. These authors described the cross-linking as polyphenols reacting under oxidising conditions with gelatin side chains forming covalent cross links, resulting in tighter chains between links. Such a structure has greater mechanical strength and thermal stability, with less capacity to expand and so absorb water. Coacervated gelatin–pectin microparticles when cross-linked became more lipophilic, due to fewer ionic groups exposed and were stable at temperatures up to 200^{o}C, in contrast to uncross-linked particles that coalesced and/or disintegrated on heating. Also, Zhang, Do, Casey, Sulistio, Qiao, Lundin, Lillford and Kosaraju (2010), cross-linked gelatine with caffeic acid or tannic acid, which resulted in the formation of insoluble hydrogels for sustained release as delivery systems.

The use of carbohydrates with proteins during microparticle preparation gives increased emulsion stability and better protection of active ingredients against oxidation (reviewed by Nesterenko et al., 2013). Whey protein in combination with carbohydrate has been used as delivery systems for volatile compounds (reviewed by Madene, Jacquot, Scher and Desobry, 2006). In such systems, whey proteins served as an emulsifying and film-forming agent while the carbohydrates (maltodextrins) acted as the matrix-forming material.

2.5 CONCLUSIONS

Protein microparticles such as KEMs have been developed and these have shown to effectively encapsulate phenolic compounds. It has also been established that the morphology and mechanical strength of protein microparticles, that in turn influence their functional performance, depend largely on the type of protein and method of preparation employed. It is possible to produce protein microparticles with improved functional properties for optimal use by cross linking. Therefore the objective of this study was to modify the functional properties of kafirin microparticles using shear and sorghum polyphenols as cross linking agents for improved encapsulation capacities.

3 HYPOTHESES AND OBJECTIVES

3.1 Hypotheses

a) A high rate of water addition (stirrer speed) during preparation of kafirin microparticles (KEMs) by coacervation, with kafirin extracted from white tan-plant and red non tannin sorghum grains will change the morphology of the KEMs. These KEMs will be smaller in size due to the high shear and turbulence introduced during formation. According to Betz and Kulozik (2011), by increasing the stirrer speed during preparation, a reduction in the microcapsule size is expected due to the stronger comminution of droplets.

b) KEMs made with 81% kafirin extracted from white tan-plant sorghum grain and prepared with low or high stirrer speeds during coacervation, will have different morphologies than KEMs made with 84% kafirin extracted from red non-tannin sorghum grain. The KEMs made with 81% kafirin at high speed, will be smaller in size and the KEMs made with 84% kafirin will be larger in size as a result of phenolic compounds (red pigments) present in red non-tannin sorghums. Taylor et al. (2009a) using kafirin extracted from a white tan-plant sorghum, described KEMs made using acetic acid as solvent, as small aggregates of different kafirin molecules, spherical or irregular in shape. According to Dykes and Rooney (2007), all sorghums contain phenolic acids. Red sorghum have higher polyphenol levels (Dykes et al., 2005), in particular flavonoids than tan-plant sorghum (Dykes et al., 2009), which can bind to kafirin as polyphenols are known to bind to protein (Siebert, Troukhanova and Lynn, 1996). Daiber and Taylor (1982) indicated that kafirin extracted from high-tannin sorghum were bound to tannins compared to non-tannin sorghum kafirin. Thus, sorghum tannins are capable of binding strongly to kafirin through hydrogen and hydrophobic bonds leading to the formation of larger complexes or aggregates (Butler et al., 1984) than kafirin extracted from non-tannin sorghum.

c) KEMs made with 81% kafirin extracted from a white tan-plant sorghum grain and prepared from aqueous red tannin sorghum phenolic extracts during coacervation, will be larger in size with higher total phenolic content and antioxidant activities

than KEMs prepared from black non-tannin sorghum phenolic extracts during coacervation. As reviewed by Duodu et al. (2003), tannins are believed to bind most strongly to kafirin, thus leading to the formation of bigger aggregates as already described**.** In sorghum, phenol contents correlates most strongly with antioxidant activity, indicating that the phenolic compounds are largely responsible for the activity (reviewed by Awika and Rooney, 2004). White sorghum varieties have very low levels of phenolics, whereas black sorghums have high levels of 3-deoxyanthocyanins, with high antioxidant activity (Awika, Rooney and Waniska, 2004b). However, according to Hagerman, Rice and Ritchard (1998) tannins in tannin sorghum, contribute higher phenolic content and antioxidant activities than simple phenolics because they have more hydroxyl groups on molar or unit basis for radical scavenging than simple phenolics, found in black and non-tannin sorghums.

3.2 Objectives

a) To determine the effects of the rate of water addition (stirrer speed) during coacervation to produce KEMs made with kafirin extracted from white tan-plant and red non-tannin sorghum grains, on their morphology (size and shape).

b) To determine the effects of kafirin extracted from different sorghum grains to produce KEMs on their morphology.

c) To determine the effects of addition of different aqueous sorghum phenolic extracts during coacervation to produce KEMs on the physico-chemical properties of the KEMs.

4 EXPERIMENTAL

4.1 Materials

A white-tan plant (WTP) sorghum cultivar (Orbit) was used for the extraction of kafirin. Sorghum flour from WTP sorghum grain was obtained from milling whole sorghum grain using a laboratory hammer mill (Falling Number 3100, Huddinge, Sweden) fitted with a 500 µm opening screen. PAN 3860 (a red condensed tannin cultivar) and black non-tannin sorghum cultivar were used for the preparation of sorghum phenolic extracts. Kafirin extracted from a red non-tannin sorghum grain (RNT) with a protein content of 84%, was kindly donated by the Council for Scientific and Industrial Research (CSIR), Pretoria, South Africa.

4.2 Methods

4.2.1 Extraction of kafirin

A method of Emmambux and Taylor (2003) was used for the extraction of kafirin. In brief, sorghum flour was mixed with 70% (w/w) aqueous ethanol containing 5% sodium hydroxide (w/w) and 3.5% sodium metabisulphite (w/w) at 70^{o}C and vigorously stirred for 1 hr to extract kafirin. After centrifugation to recover the supernatant, the ethanol was evaporated off. The protein suspension was adjusted to pH 5.0 to precipitate kafirin, filtered under vacuum and freeze-dried. Defatting of the kafirin with hexane was then done at ambient temperature and the kafirin was air-dried. The protein content of the WTP sorghum kafirin was 81% (N x 6.25, dry matter basis).

4.2.2 Extraction of aqueous phenolics from sorghum bran

PAN 3650 (condensed tannin sorghum) and black non-tannin sorghum grains were used. Equal amounts of cleaned grain (75 g) were each placed into eight sample cups and decorticated for approximately 2 min according to a method of Reichert, Tyler, York, Schwab, Tatarynovich and Mwasara (1986) using a Tangential abrasive dehulling device (TADD) fitted with 60 grit paper (Norton Metalite R2P4) to give a bran yield of approximately 8%. The collected sorghum brans were then sieved through a 1000 µm aperture sieve and ground into a powder using a coffee grinder. The powder was further sieved through a 500 µm aperture sieve, packed under vacuum and stored at 4^{o}C.

In brief, aqueous phenolic extracts were prepared from sorghum brans with distilled water adjusted to pH 2.0 according to a method of Liyana-Pathirana and Shahidi (2005) with some modifications. After continuously stirring the sorghum bran in 150 ml distilled water for 30 min, pH was adjusted to pH 2.0 using 1 M HCI and then incubated in a shaking water bath at 37^{o}C for 30 min. After this, the pH was further adjusted to pH 6.0 and incubated for another 30 min. The aqueous extracts were then centrifuged at 7500 g for 5 min and the respective supernatants decanted into plastic containers wrapped in foil and frozen until further analysis.

4.2.3 Preparation of kafirin microparticles

A method according to Taylor et al. (2009a) with slight modification was used to prepare kafirin microparticles by coacervation with 81% (WTP) and 84% (RNT) protein kafirins. Two grams of 81% kafirin and 1.9 g of 84% kafirin were weighed and mixed in 5 ml glacial acetic acid with gentle stirring. To allow for equilibration, the solution was allowed to stand for 16 h. After full solvation of kafirin, the coacervation was done by adding distilled water at ambient temperature at two different rates (0.7 ml/min and 1.4 ml/min) respectively, using a Watson-Marlow Bredel peristaltic pump (Falmouth, England) to kafirin solution, stirring at 600 rpm using a magnetic stirrer. The resulting concentration of the suspension was 2% (w/w) kafirin and 5.4% (w/w) acetic acid. Some of the kafirin microparticles were kept as wet samples for further analysis. Dry microparticles were obtained by washing in distilled water three times to remove acetic acid by centrifugation at 1100 g for 5 min. The resulting pellets were then freeze-dried and stored at 10^{o}C.

4.2.4 Preparation of kafirin microparticles with aqueous sorghum phenolic extracts

The phenol-bound kafirin microparticles were prepared with 81% (WTP) protein kafirin from the different sorghum phenolic extracts as described in 4.2.3 with modifications. The total phenolic contents of the black non-tannin and red condensed tannin sorghum phenolic extracts were 4.6 and 10.1 mg CE/10 mg (catechin equivalent, dry basis), respectively. During preparation of microparticles, the water used for coacervation was substituted with different concentrations of aqueous sorghum phenolic extracts at an addition rate of 1.4 ml/min. For the red tannin extracts the following concentration of extract-to-water during coacervation were used: 20, 40 and 100% (v/v), whereas for black non-tannin sorghum

phenolic extracts, 40, 50 and 100% (v/v) were used. Higher concentrations were used for the black non-tannin phenolic extracts as the total phenolic content of this extract was lower than that of the red condensed tannin phenolic extract.

4.2.5 Protein

The protein content (N x 6.25) of the kafirin microparticles was determined by a Dumas combustion method, American Association of Cereal Chemists (AACC, 2000) Approved Method 46-30.

4.2.6 SDS-PAGE

The different kafirins and kafirin microparticles were characterized by sodium dodecyl sulphate polyacrylamide gel electrophoresis procedure (SDS-PAGE) as described by Taylor, Bean, Ioerger and Taylor (2007) on a 4-18% acrylamide gradient gel under both reducing and non-reducing conditions. Invitrogen Mark 12 unstained molecular marker (Invitrogen Life Technologies, Carlsbad, CA) was used as the molecular standard. The protein loading was approximately 10 µg per lane.

4.2.7 Microscopy

Light microscopy

The general sizes and shapes of the kafirin microparticles were determined by viewing the wet samples with a Nikon Optiphot light microscope (Kanagawa, Japan).

Electron microscopy

The external morphology of dry kafirin microparticle samples was observed using a scanning electron microscope (Joel JSM-840, Tokyo, Japan) and the internal morphology of the wet kafirin microparticles was determined using a transmission electron microscope (Joel JEM-2100F Field Emission Electron Microscope, Tokyo, Japan) according to Taylor et al. (2009a).

4.2.8 Size distribution of kafirin microparticles

The size of the kafirin microparticles was determined by comparing their images using a scale bar. Duplicate measurements of each treatment were made with a count of at least 100 microparticles each.

4.2.9 Total phenolics

The total phenolic content of phenol-bound kafirin microparticles was determined using the Folin-Ciocalteu method (Singleton and Rossi, 1965) with modification. Bound phenolics in the microparticles were released by hydrolysis. Briefly, the hydrolysis was carried out by adding 5 ml 2 M NaOH to 200 mg of respective phenol-bound kafirin microparticles. The mixture was allowed to stand at ambient temperature for 3 h. After this, the pH was then adjusted to pH 7.4 using either 1 M HCI or 1 M NaOH. The samples were then centrifuged at 7500 g for 5 min. The clear supernatant was then assayed. Catechin was used as a standard.

4.2.10 Antioxidant activity

The free radical scavenging activity of the phenol-bound kafirin microparticles was determined using the Trolox Equivalent antioxidant capacity (TEAC) assay ($ABTS^{\cdot+}$ free radical scavenging) as described by Awika, Rooney, Wu, Prior and Zevallos (2003) with modifications. The ABTS radical cation ($ABTS^{\cdot+}$) was generated by mixing equal volumes of 8 mM ABTS (2, 2`-azino-bis-(3-ethyl-benzothiazoline-6-sulphonic acid)) salt and 3 mM potassium persulphate ($K_2S_2O_8$) using deionised water and allowed to react in the dark for 12-16 h. The $ABTS^{\cdot+}$ solution was then buffered pH 7.4. Hydrolysed extract samples (prepared as described in 4.1.2.9) were further diluted 1 in 10 for black non-tannin extracts and 1 in 20 for red tannin extracts with phosphate buffer pH 7.4 before assay. Trolox was used as standard.

4.2.11 Statistical analyses

The experiments were repeated at least once and carried out in triplicate unless stated otherwise. The resulting data were analysed using one way analysis of variance (ANOVA).

The means were compared using Fisher's least significant difference (LSD) test. The computations were performed using Statistica Version 8.0 (Statsoft, Tulsa, USA).

5 RESULTS AND DISCUSSION

5.1 Effects of kafirin source and speed of water addition during coacervation on the shape and size of kafirin microparticles

The preparation of kafirin microparticles with kafirin extracted from a white tan-plant (WTP) Orbit and red non-tannin (RNT) sorghum types of 81% and 84% protein content respectively and the different speeds of water addition used during coacervation resulted in kafirin microparticles (KEMs) of very different size and shape. Water flow rates of 1.4 ml/min and 0.7 ml/min during coacervation using 81% kafirin from WTP sorghum resulted in mostly small spherical-shaped KEMs (white KEMs) (Figure 5: a, b; light microscopy (LM)), with rough porous surfaces as shown by scanning electron microscope (SEM) (Figure 5: a, b) and internal vacuoles (roughly under 2 µm in size) (Figure 5: a, b; transmission electron microscope (TEM)). The diameters of white KEMs ranged between 1 to 10 µm (Figure 6: a, b). Taylor et al. (2009a) and Anyango et al. (2012) described similar morphologies for the KEMs prepared with kafirin extracted from WTP sorghum.

However, the KEMs made with 84% kafirin from RNT sorghum (red KEMs) were very different from the white KEMs in terms of shape and size. The red KEMs were mainly oval in shape as shown by LM (Figure 5:c, d) and appeared to have formed larger aggregates (clusters) of microparticles with more numerous larger vacuoles of varying sizes (up to 5 µm) as shown by TEM (Figure 5:c, d). The surface edges ranged from smooth to rough surfaces as shown by SEM (Figure 5:c, d). At a flow rate of 1.4 ml/min during caocervation, the red KEMs had an average length and width of 91 and 30 µm and at a flow rate of 0.7 ml/min during coacervation, the length and width were approximately 43 and 21 µm respectively (Figure 6: c, d). The particle size distribution were very wide for the red KEMs; the majority of red KEMs produced at slow speed ranged from 31 to 50 µm and those produced at high speed 51 to 100 µm (Figure 6). Thus, at high speed during formation, bigger microparticles were formed.

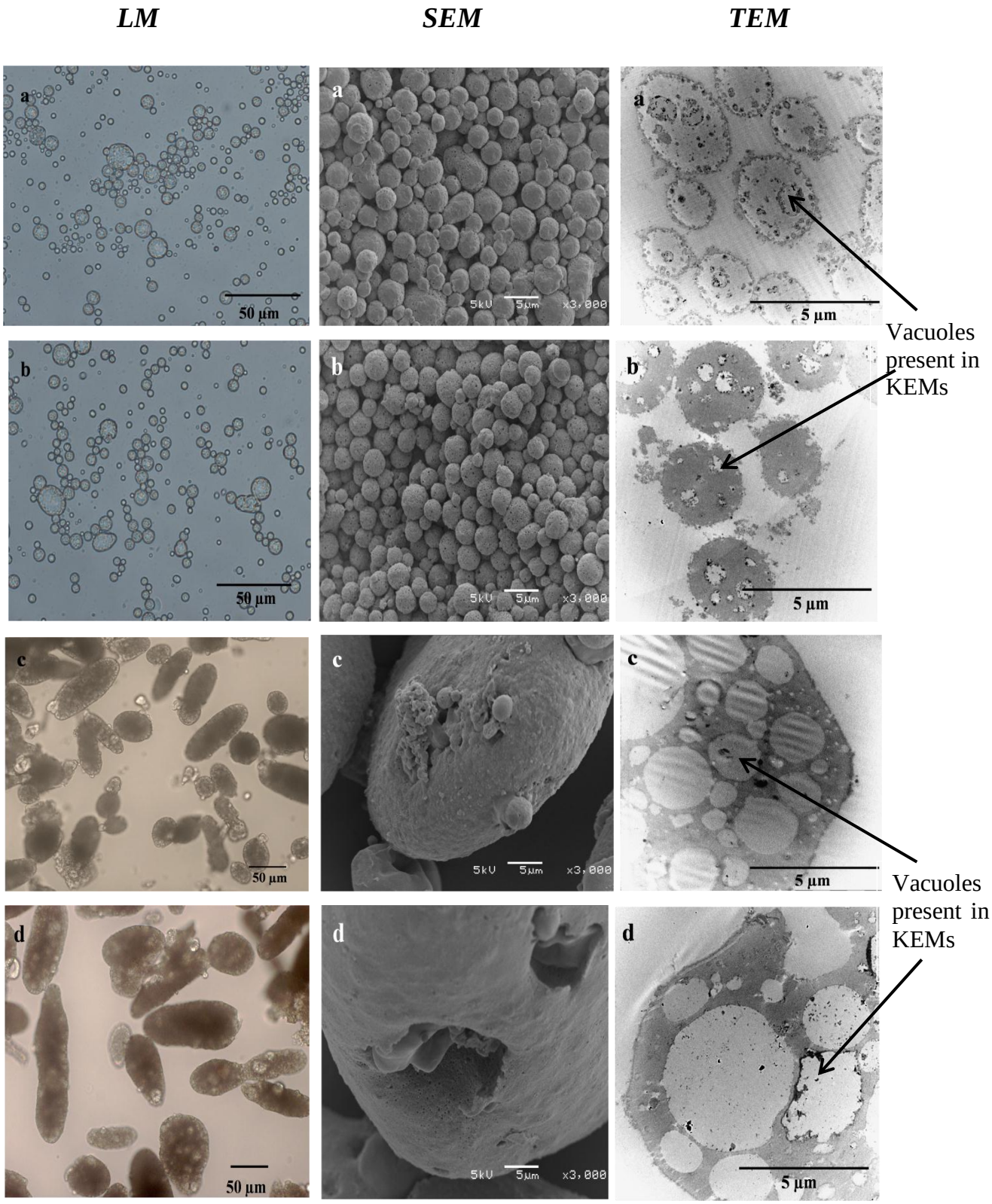


Figure 5: Micrographs of kafirin microparticles made from white and red sorghum kafirin. a: white KEMs at 0.7 ml/min, b: white KEMs at 1.4 ml/min, c: red KEMs at 0.7 ml/min, d: red KEMs at 1.4 ml/min

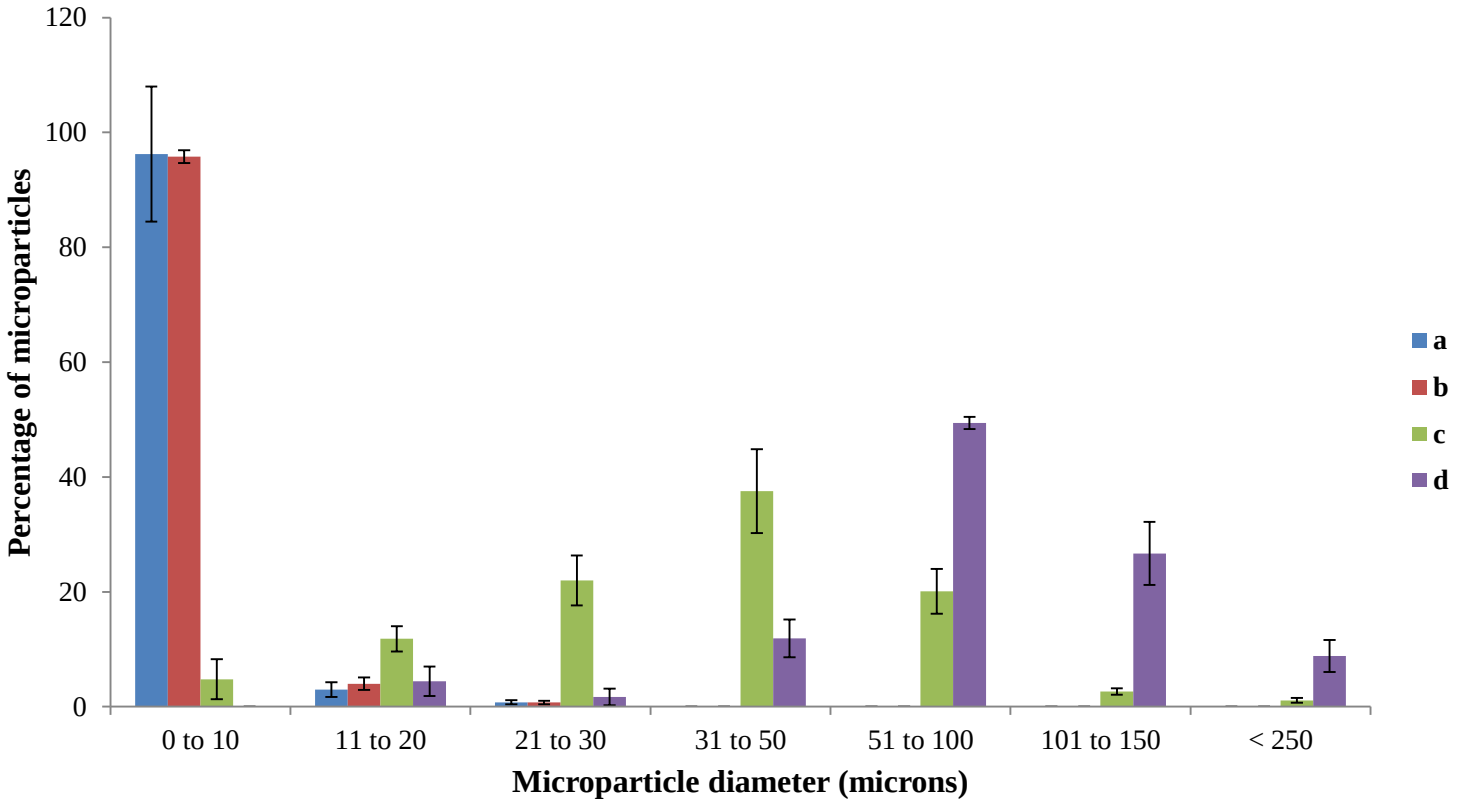


Figure 6: Particle size distribution of white KEMs (WK) and red KEMs (RK)

a-white KEMs at 0.7 ml/min, b-white KEMs at 1.4 ml/min, c- red KEMs at 0.7 ml/min, d- red KEMs at 1.4 ml/min
Standard deviations are given in error bars, n = 3

Throughout literature, the effect of stirrer speed on the size and shape of microspheres is not consistent (Arshady, 1990; Esposito, Cortesi and Nastruzzi, 1996; Zhao, Gagnon and Häfeli, 2007; Taylor et al., 2009a and reviewed by Matalanis et al., 2011). Research has shown that smaller coacervate particles are formed at higher stirring speeds (reviewed by Matalanis et al., 2011). Betz and Kulozik (2011) stated that by increasing the stirrer speed during microparticle preparation, a reduction in the microcapsule size is expected due to the stronger comminution of droplets. However, there is a limit to this effect as excessively high shear rates can result in protein microparticle aggregation (reviewed by Matalanis et al., 2011). In this study, however, it was a different case. The presence of bigger particles at high speed could have been due to the shorter mixing time during preparation.

Lehtinen and Zachariah (2001) who studied the effect of coalescence energy release on the temporal shape evolution of nanoparticles, attributed the formation of an oval shape to the rate of coalescence being greater than the rate of collision. These authors found that a neck is formed between two particles when they coalesce. New chemical bonds that are formed result in energy release and therefore an increase in particle temperature which rapidly speeds up the coalescence. Thus, an oval shape is then formed. Anyango et al. (2012) described the microparticles formed following a wet heat treatment as oval shaped and larger in size compared to the original KEMs, which were smaller and attributed the change in shape to the rate of particle coalescence being inversely proportional to the particle size. Thus the oval shaped KEMs produced by the red KEMs could be due to the same effect.

Taylor et al. (2009a) in their preparation of KEMs attributed that the presence of vacuoles or internal holes in the microparticles possibly to footprints of air bubbles entrapped during coacervation. They suggested that, since the solution of kafirin dissolved in glacial acetic acid used in the preparation of KEMs is viscous with air bubbles present, considerable foaming would occur when water is added during coacervation. Thus it was possible that air bubbles may be entrapped in the microparticles during formation and then appear as holes or vacuoles in the ‘set’ microparticles as observed by TEM (Figure 5). The red KEMs could possibly also entrap in more air during coacervation due to their bigger size, resulting in bigger vacuoles. The conditions and method of preparation affects the size and properties of microparticles (Sinha and Trehan, 2003). Hence, to establish the reasons for the differences in KEMs morphology, the protein content and SDS-PAGE were determined

to see whether the source of kafirin (WTP or RNT) and its composition had an influence on the average size (5 µm compared to 100 µm) of the white and red KEMs, respectively.

5.2 Protein content and SDS-PAGE of kafirin and kafirin microparticles

The protein content of the different kafirin and KEMs were determined. As shown in Table 2, white KEMs made with 81% kafirin had similar protein content at the two rates of water addition. However, the protein content of the white KEMs were higher than red KEMs made with 84% kafirin at the two rates of water addition.

Table 2: Protein content of kafirins and microparticles (g/100 g) dry basis

Materials	Protein content
Sorghum type	
White tan-plant	81^{A} (0.1)
Red non-tannin	84^{*B}
Kafirin microparticles	
White KEMs (0.7 ml/min)	79.6^{c} (0.3)
White KEMs (1.4 ml/min)	79.3^{c} (0.2)
Red KEMs (0.7 ml/min)	74.1^{b} (0.3)
Red KEMs (1.4 ml/min)	72.5^{a} (0.5)

Values with different letters in the same column are significantly different ($p < 0.05$)
Upper case letters are used for the sorghum, lower case letters for kafirin microparticles
Standard deviations are given in parentheses, n = 3
*Provided by the Council for Scientific and Industrial Research (CSIR)

It seems that the kafirin with higher protein content (84%) had lower protein content (74.1%) in their respective microparticles. This could possibly mean less kafirin was available for the formation of red KEMs than for white KEMs despite the fact that red non-tannin kafirin had the higher protein content. The difference in protein values for the red KEMs could probably be due to impurities introduced during the washing of the wet samples for freeze drying.

SDS-PAGE of kafirin and kafirin microparticles made from WTP and RNT sorghum kafirin (81 % and 84 % protein, respectively) were performed under reducing and non-reducing conditions. The band pattern for non-reducing and reducing conditions was the same except that the bands under reducing conditions for the white kafirin and its KEMs were slightly darker than for the red kafirin and its microparticles in spite of loading to constant protein content. Molecular weights of 18k, 22-31 kDa were present as shown in (Figure 7) below.

Taylor et al. (2009a) also reported bands in the same range and according to El Nour, Peruffo and Curiono (1998), these bands could probably be equivalent to the γ- (26 k), α1- (26 k) and α2- (22 k) bands. Thus the cconditions of the microparticle preparation did not seem to have an apparent effect on the kafirin proteins.

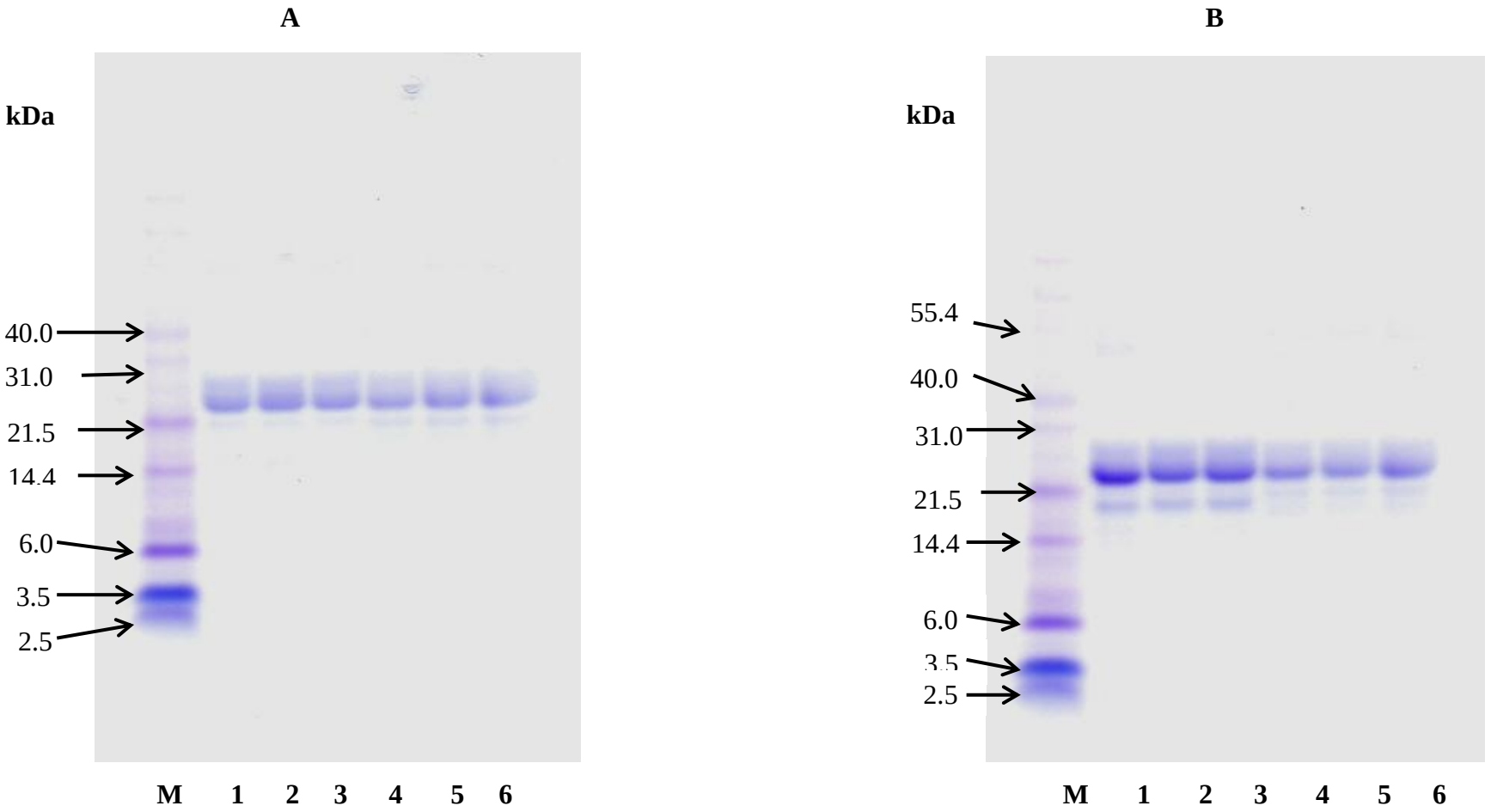


Figure 7: SDS-PAGE of kafirin from white and red sorghums and their microparticles.

(A) non-reducing conditions (B) reducing conditions. M: Molecular weight marker; 1: kafirin from white sorghum, 2: White KEMs at 0.7 ml/min, 3: White KEMs at 1.4 ml/min, 4: kafirin from red sorghum, 5: Red KEMs at 0.7 ml/min, 6: Red KEMs at 1.4 ml/min

Since the source of kafirin in relation to the sorghum cultivar did not have an effect on the morphology of KEMs formed with regard to size, this lead to a hypothesis that the presence of phenolic compounds (red pigments) in the red non-tannin sorghum could be responsible for the larger and oval-shaped red KEMs compared to the small, spherical white KEMs. The phenolic compounds are known to form complexes with kafirin in sorghum (Emmambux and Taylor, 2003).

5.3 Effect of sorghum phenolics on microparticle morphology

The morphology and size of KEMs prepared with 81% kafirin (white KEMs) extracted from WTP sorghum and varying concentration of black non-tannin and red tannin sorghum phenolic bran extracts used during coacervation were determined. Overall, there was a change in shape with increased particle and vacuoles size. As already determined, the white KEMs prepared with 0% sorghum phenolic extracts were small and spherical (Figure 8: a, LM), with an average diameter of 5 µm (Figure 9: control) and vacuoles size of > 2 µm (Figure 8: a, TEM). When the black non-tannin phenolic extracts were substituted in varying concentration (40, 50 and 100%) for the water used for coacervation, there was a change in KEMs morphology. As the concentration of black non-tannin phenolic extract increased, the KEMs became larger in size up to < 18 µm average diameter (Figure 8: d) with an increase in vacuole size (< 5 µm) (Figure 8: d, TEM). However, the change in morphology of KEMs was more prominent in KEMs prepared using red tannin phenolic extracts during cocervation at different concentrations (20, 50 and 100%). There was a progressive and large change in KEMs size and shape as shown in Figure 8: e, f, g by LM (size and shape), SEM (external surface) and TEM (internal surface) respectively. Even at low red tannin phenolic concentration (20 %), the average particle size was four times more (Figure 8: e) than the control and they seemed to maintain their spherical shape. However at 50% red tannin phenolic concentration, the number of spherical microparticles decreased and appeared as large oval-shaped microparticles with a very wide size distribution (Figure 9: f). At 100% tannin concentration, the microparticles appeared to have formed very large clusters of few oval-shaped microparticles. The highest length and width recorded on these KEMs was approximately 400 µm and 90 µm respectively (Figure 8: g, LM). The numbers of vacuoles on their surface were even larger > 5 µm and more numerous as shown by the TEM (Figure 8: g).

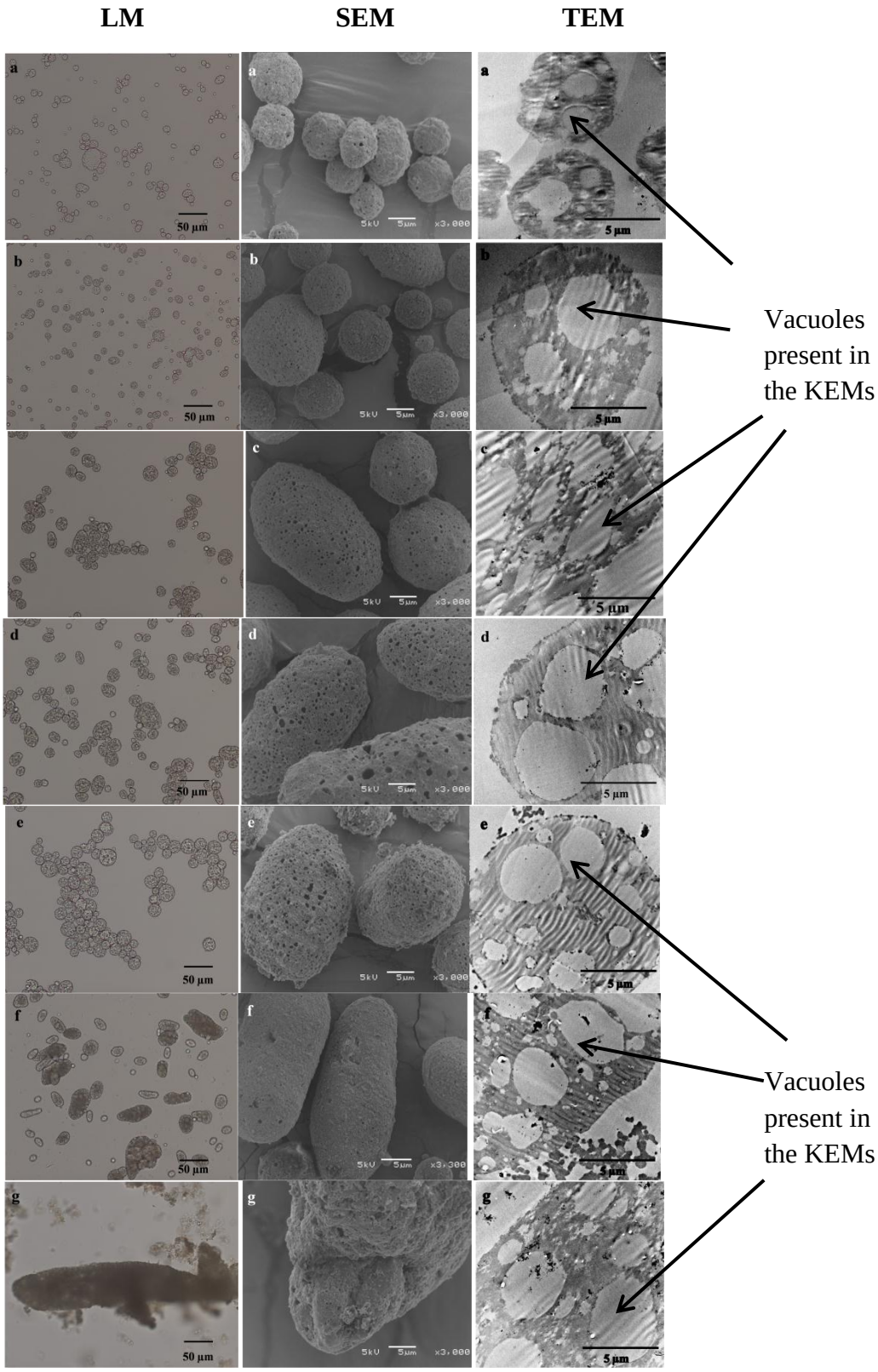


Figure 8: Micrographs of kafirin microparticles prepared with 81% kafirin WTP and aqueous sorghum black non-tannin (BNT) and red tannin (RT) phenolic extracts. a:Control- 0 % phenolic extract, b:40% BNT phenolic extracts , c:50% BNT phenolic extracts , d:100% BNT phenolic extracts , e:20% RT phenolic extracts , f:50% RT phenolic extracts , g:100% RT phenolic extracts.

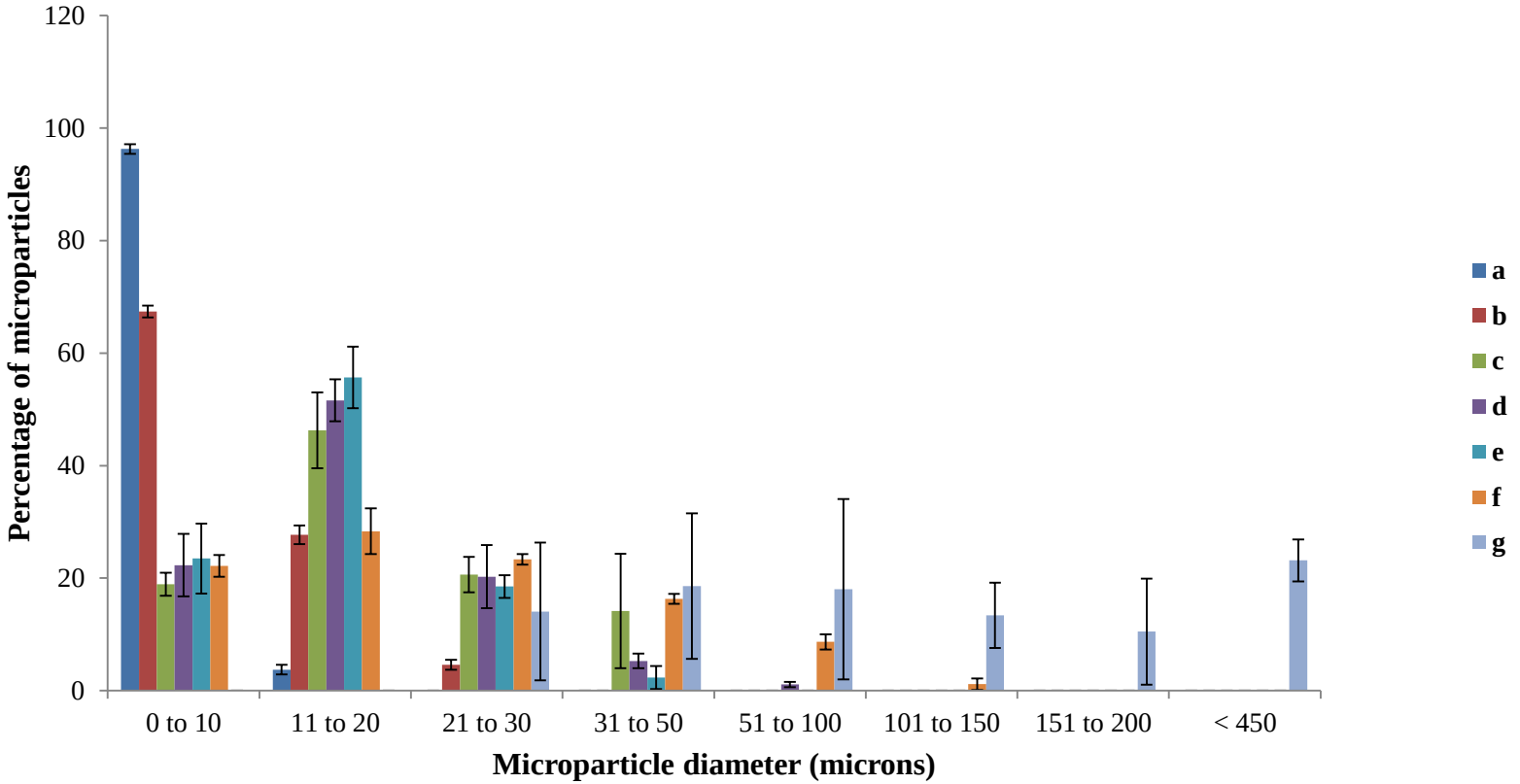


Figure 9: Particle size distribution of KEMs prepared with 81 % kafirin and aqueous sorghum phenolic extracts.

a: control- 0 % phenolic extract, b: 40% - Black non-tannin phenolic extracts (BNT), c: 50% BNT, d: 100% BNT , e: 2.5% Red tannin (RT), f: 5% RT, g: 100% RT phenolic extracts.

Standard deviations are given in error bars, n = 3

According to Dykes and Rooney (2007), all sorghums contain phenolic acids and most contain flavonoids but only varieties with a pigmented testa have condensed tannins. Sorghums with a black pericarp are known to have the highest level of 3-deoxyanthocyanins (Dykes et al. 2005). Of all the phenolic compounds present in sorghum, condensed tannins are known to form irreversible complexes with kafirin due to their high affinity for the sorghum protein (Emmambux and Taylor, 2003). Thus the phenolic extracts, depending on their molecular size and affinities for kafirin can result in KEMs with different morphologies by binding to kafirin.

Tannins have a high binding affinity for kafirin (Butler et al., 1984). In general, proteins that bind sorghum tannin strongly are relatively large, have a loose, open structure, and are rich in proline. Kafirin, the sorghum prolamin storage protein, is relatively rich in proline (11 mole %) (Evans, Schüssler and Taylor, 1987) and has shown to form complexes with both sorghum condensed tannins and tannic acid (Emmambux and Taylor, 2003). All of these characteristics maximize the opportunity for forming multiple hydrogen bonds between tannin molecules and the peptide backbone of the protein for non-polar interactions.

Also, sorghum grain protein content varies from 6 to 18%, with an average of 11% protein (reviewed by Mesa-Stonestreet, Alavi and Bean, 2010) and thus in theory; high tannin cultivars would contain more than enough tannin (2-4%) to bind all the seed protein. It is believed that under optimal conditions, sorghum tannins are capable of binding and precipitating at least 12 times their own weight of protein and the tannin-protein interaction in sorghum is thought to involve hydrogen bonding and non-polar hydrophobic interactions (Butler et al., 1984).

Lower protein yields were obtained for high-tannin compared with low-tannin (condensed tannin-free) sorghum on subjecting both grains to the Landry-Moureaux protein fractionation procedure, in a work performed by Daiber and Taylor (1982). According to these authors, this was due to interactions between tannin and the sorghum protein, rendering the proteins insoluble. Further work by electrophoresis revealed that proteins extracted from high tannin sorghum were bound to tannins.

This could explain why bigger KEMs were formed when red tannin phenolic extracts were used during coacervation to form KEMs. Due to the multiple hydroxyl groups, tannins may interact with and form complexes with kafirin which may lead to precipitation because of the large size of tannins (reviewed by Duodu et al., 2003), resulting in bigger KEMs compared to other sorghum phenolic compounds. Because of the bigger complexes, more air can possibly be trapped in these complexes giving rise to bigger vacuoles in the resulting microparticles. The mechanism of formation will be discussed later on.

5.4 Phenolic content and antioxidant activity of kafirin

The total phenolic content (TPC) and antioxidant activity (AA) of the KEMs made with 81 % kafirin from varying concentration of aqueous black non-tannin and red tannin sorghum bran phenolic extracts are summarised in Table 3.

The TPC and AA of KEMs made from black non-tannin phenolic extracts were not significantly different from the KEMs made with 0 % sorghum phenolic extracts. Thus black non-tannin phenolic extracts did not contribute significantly towards the TPC and AA of the KEMs. However, a noticeable difference was seen with the KEMs made from red tannin phenolic extracts. As the concentration of the red tannin phenolic extract increased in the preparation of KEMs during coacervation, a progressive increase in the TPC and AA was recorded, from 0.1 to 28.8 mg CE/100 mg and 348 to 862 µMTE/100 mg, respectively.

Table 3: Phenolic content and antioxidant activity of KEMs with aqueous sorghum phenolic extracts

KEMs	Concentration of sorghum phenolic extracts (%)	Total phenolics (mg CE/100 mg)	Antioxidant activity (µMTE/100 mg)
Control			
	0	0.1[a] (0.0)	348[a] (47)
Non-tannin black sorghum phenolic extracts			
	40	0.5[ab] (0.1)	381[a] (5)
	50	0.9[bc] (0.1)	361[a] (55)
	100	1.0[c] (0.0)	425[ab] (46)
Red-tannin phenolic sorghum extracts			
	20	14.1[d] (0.6)	468[b] (2)
	40	16.6[e] (0.6)	506[b] (4)
	100	28.8[f] (0.5)	862[c] (44)

Mean values within a column with different letters differ significantly ($p < 0.05$).

Standard deviations are given in parentheses, n = 3

So far in literature there is no work reported on the AA of KEMs except the work done by Taylor et al. (2009b). These authors encapsulated kafirin microparticles with catechin and condensed tannin and reported to have AA of 15327 and 5366 µmol of Trolox equiv/g respectively. However according to Pẻrez-Jimènez and Saura-Calixto (2006), large variability in antioxidant activities is expected due to the different methods of analysis used and the solvent applied to dissolve the polyphenol.

In sorghum, phenol content correlates most strongly with antioxidant activity, indicating that the phenolic compounds are largely responsible for the activity (Awika et al., 2003; Dykes et al., 2005). Hence, the higher the TPC, the higher the AA values as shown in Table 3.

White sorghum varieties have very low levels of flavonoids, and the bound phenolic acids are the most important source of AA, whereas black sorghums have more than twice the level of 3-deoxyanthocyanins than other sorghum varieties, which may also contribute to the AA (Awika, Rooney and Waniska, 2004a). According to Hagerman et al. (1998), condensed tannins in tannin sorghums, contribute higher AA than simple phenolics because they have more hydroxyl groups on molar or unit basis for radical scavenging than simple phenolics. Also, these sorghums have consistently shown the highest AA in vitro (Dykes and Rooney, 2007). Thus, the high TPC and AA in red tannin KEMs could possibly be attributed to condensed tannins, believed to be present in the red tannin phenolic extracts used for the formation of these KEMs.

According to Riedl and Hagerman (2001), condensed tannins complexed with proteins retain at least 50% of their antioxidant activity which acts as a free radical sink within the gastrointestinal tract, thus sparing other antioxidants. Therefore, it is possible that the additional antioxidative value of the KEMs cross-linked with condensed tannins would also be available to act as a free radical sink within the gastrointestinal tract as also suggested by Taylor et al. (2009b).

5.5 CONCLUSIONS

Phenolic compounds present in sorghum are responsible for the shape and size of microparticles formed. The extent of KEMs modification depends on the type and amount of polyphenols used for cross-linking. Therefore sorghum polyphenols can be used to cross link kafirin in the preparation of microparticles with an improvement in their functional properties such as loading capacities. Of the sorghum polyphenols, condensed tannins are the most beneficial as they contribute the most towards the AA of the KEMs, resulting in the development of innovative KEMs with added antioxidative benefits.

6 GENERAL DISCUSSION

This chapter is divided into three parts. The first part will critically evaluate assay methods used in this study namely, Microscopy, Folin-Ciocalteu phenol and Trolox equivalent antioxidant capacity assays. The main aim is to reveal the strengths and weakness of these methods as they were applied. The second part will propose a model for the mechanism of cross- linking kafirin with polyphenols in manipulating the morphology of the resultant kafirin microparticles during coacervation, with particular reference to condensed tannins (CT) and 3-deoxyanthocyanins. The last section will briefly look at future research and potential applications of these cross linked kafirin microparticles with polyphenols.

6.1 Methodology: critical review

In this study, the morphology of the KEMs were largely characterised by three types of microscopy. According to Taylor (2008), all types of microscopy involve precision optical instruments that are sensitive, expensive to purchase and maintain. The use of glacial acetic acid in the preparation of KEMs could generally result in corrosive materials and hence may corrode the instruments. Thus, the visualisation of KEMs as wet sample preparations was only limited to light microscopy (LM) at lower magnification than scanning and transmission electron microscopy. A magnification of x 200 was used to view and photograph the KEMs in this study, although higher magnifications could have given more detailed information on the formation of KEMs. However, according to Yang, Wang, Lai, An, Li and Chen (2007), despite the use of low magnification and resolution that gives the viewer only two dimensional images, LM has the advantages of being relatively fast, cheap and allowing for a large scan area.

The external morphology of KEMs was viewed using scanning electron microscopy (SEM). As SEM operates by allowing a focused electron beam to be scanned across the sample whilst under vacuum (Casuccio, Schlaegle, Lersch, Huffman, Chen and Shah, 2004), samples have to be analysed dry to avoid sample destruction in the microscope chamber. Thus, the wet KEMs preparations had to be freeze-dried before analysis, which had a great potential of introducing

artefacts as some images after analysis appeared clumped together. Also at high magnification of x 3000, there was poor resolution of the samples and thus to enable comparison, magnification was limited to x 3000 for all samples. Taylor (2008) and Anyango (2012) also reported that sample preparation of KEMs for SEM can produce artefacts which may harbour various risk factors that can easily distort the integrity and ultra-structure of the samples making scientific interpretation difficult. However, according to Stadtländer and Kirchhoffa (2004) and Standtländer (2007), generally the use of SEM gives the viewer the impression of three dimensions making it easier to interpret the images. It also allows viewing of whole sample (instead of sample sections) and the procedure is relatively less time consuming and simple compared to transmission electron microscopy (TEM) which was also used in this study, in order to view the internal morphology of KEMs.

TEM projects electrons through an ultrathin slice of specimen and produces a two-dimensional image (Casuccio et al., 2004). As a result of extensive sample preparation for TEM, the problem of artefacts was also unavoidable. TEM artefacts are particularly significant if one has to get a clear understanding of the cross-linking behaviour of polyphenols and kafirin in microparticle preparation. For this TEM investigation, the wet kafirin microparticle samples were dehydrated with acetone instead of ethanol, which is normally used (Standtländer, 2007), since kafirin dissolves in 70% aqueous ethanol (Taylor, 2008). Also, the embedding of samples in resin appeared unsuccessful and as a result during sectioning, some resin blocks were shattered during cutting. This could have an effect of altering the integrity of the final electron micrographs as samples were held within the resin. Thus, propylene oxide (1, 2-epoxypropane) was added soon after the dehydration step with acetone and before infiltration with resin and embedding the samples, to allow for effective hardening of the resin. According to Standtländer (2007), the replacement of the dehydration solution, in this case acetone, by another intermediary solvent (propylene oxide) before attempting to embed the samples in a resin is crucial as acetone is not miscible with the resin. The use of propylene oxide will enable effective infiltration of samples with resin, as resin dissolves in propylene oxide. Despite the extensive sample preparatory steps required in TEM and visualisation of images in two dimensions, TEM has the advantage of high resolution with a relatively fast scan speed (Standtländer, 2007; Yanget al., 2007).

The Folin–Ciocalteu phenol reagent was used in this study to estimate the quantity of phenolics bound in the KEMs. Phenolic compounds undergo a complex redox reaction with phosphotungstic and phosphomolybdic acids present in the reagent (Wong et al., 2006), which produces coloured complexes that are easily detected spectrophotometrically with high sensitivity. The phenolic extracts used to prepare KEMs (extracted from sorghum brans) were not 100% pure as they were used directly without purification. Thus, the phenolic content measured by the Folin–Ciocalteu reagent did not give a full picture of the quantity or quality of the phenolic constituents in the extract as the total phenolic content could have been overestimated. According to Liyana-Pathirana and Shahidi (2005); Macdonald-Wicks, Wood and Garg (2006); Robbins, Kwik-Uribe, Hammerstone and Schmitz (2006), the Folin–Ciocalteu reagent is not specific to polyphenols as it can detect reducing groups in other non-phenolic compounds including amino acids, ascorbic acid and reducing sugars, which are commonly found in food matrices.

The Trolox equivalent antioxidant capacity (TEAC) assay was used to measure the antioxidant activity of the phenolics bound in the KEMs. This assay measures the relative ability of antioxidant substances to scavenge the 2, 2'-azinobis (3-ethyl-benzothaizoline-6-sulphonic acid) radical cation chromogen ($ABTS^{\cdot+}$) in comparison with the antioxidant potency of a standard amount of Trolox, a water soluble analogue of α-tocopherol (reviewed by Rice-Evans, Miller and Paganga, 1997; Prior, Wu and Schaich, 2005). As reviewed by Rice-Evans et al. (1997), phenolics such as phenolic acids, flavonoids and tannins do not exert the same antioxidant activity; some may demonstrate strong antioxidative properties while others may demonstrate poor activity. They may also display antagonistic or synergistic effects among themselves or with other constituents in the extracts (Rice-Evans et al., 1997; Robbins, 2003). Thus, the antioxidant activity of the extracts may originate from the combined action of phenolic constituents. According to Dykes and Rooney (2007), all sorghums contain phenolic acids which could have also contributed to the total antioxidant activity of the phenolics bound in KEMS, giving an overestimate. However, the advantage of TEAC assay is that it is operationally simple, can be used over a wide pH range and is not affected by ionic strength thus can be used in multiple media to determine both hydrophilic and lipophilic antioxidant capacities of extracts (Prior et al., 2005).

6.2 Mechanisms of manipulating the morphology of kafirin microparticles using polyphenols

To explain the mechanism of cross-linking kafirin in glacial acetic acid solution with sorghum polyphenols extracted from tannin (condensed tannins) and black non-tannin (3-deoxyanthocyanins) sorghum brans, a model is proposed.In brief, the formation of large cross-linked KEMs is proposed to involve the binding of polyphenols to KEMs and possibly kafirin polypeptides, followed by the aggregation of the KEMs/polyphenol complex into large complexes, which then precipitate out as large cross linked KEMs. A number of authors have come out with models to explain the interaction of proline-rich proteins, like kafirin, with polyphenols (Calderon, Van Buren and Willard, 1968; Butler et al., 1984; Baxter, Lilley, Haslam and Williamson, 1997; Emmambux and Taylor, 2003; Jöbstl, O'Connell, Fairclough and Williamson, 2004; Frazier, Deaville, Green, Stringano, Willoughby, Plant and Mueller-Harvey, 2010). The same reasoning is applied for this study.

Kafirin polypeptide dissolved in glacial acetic acid exists in a partially unfolded conformation, linked with acetic acid through hydrophobic interactions (Taylor, 2008). Such an unfolded conformation is in agreement with Li, Li, Xia, Zhang, Wang and Huang (2012) who studied the dissolution behaviour of zein polypeptides in ethanol and acetic acid solutions. These authors reported that zein polypeptides appeared as partially unfolded or in an elongated conformation in both solutions. Since kafirin and zein have a large degree of homology (DeRose et al., 1989), the kafirin polypeptides are assumed to take up the same conformation in acetic acid solution.

As the polyphenols in water are added during coacervation to the solution of kafirin in acetic acid, KEMs will be formed simultaneously as they also become cross-linked with the polyphenols. However, it is possible that the polyphenols can also bind to kafirin polypeptides, which aggregate and form large KEMs also cross-linked with polyphenols. Thus, kafirin is released from its hydrophobic interaction with acetic acid and interacts with water through the amide groups of the kafirin glutamine. The polyphenols present in solution will therefore cross link the KEMs resulting in larger complexes.

Polyphenols, in particular condensed tannins (CT), have a high binding affinity to proline-rich proteins such as kafirin and are able to form strong hydrogen bonds between their hydroxyl groups and the carbonyl groups of the protein peptide bond (Butler et al., 1984; Emmambux and Taylor, 2003). Because of its partially unfolded nature, the carbonyl oxygen of the peptide bond in kafirin polypeptides are more exposed and available for hydrogen bonding than those of a compactly folded protein (Hagerman and Butler, 1981). Jöbstl et al (2004) reported that simultaneous binding of polyphenols to several sites in the proteins lead to coiling of the protein around the polyphenol. Also, Charlton, Baxter, Khan, Moir, Haslam, Davis and Williamson (2002), who studied the polyphenol/peptide binding and precipitation, suggested that longer peptides are able to wrap around polyphenols to interact at several places at once. As CT are large multidentate ligands able to bind simultaneously via different phenolic groups at more than one point on the protein strand (Charlton et al., 2002), they are able to cross-link adjacent polypeptides (McManus, Davis, Beart, Gaffney, Lilley and Haslam, 1985).

Thus, an assumption is made that as the kafirin polypeptides coil around CT, not only do polyphenols complex with one kafirin molecule at various sites but they are also able to cross link neighbouring kafirin molecules through hydrophobic interactions on the surface of the proteins. According to Charlton et al. (2002), the binding is largely at exposed hydrophobic and planar side chains dominated by the stacking of polyphenolic rings onto the planar hydrophobic surfaces. It could be expected to occur especially when the concentration of polyphenol increases during coacervation, until all binding sites are saturated.

However, in this study the polyphenols seemed to be cross-linking the already formed KEMs together forming larger cross-linked aggregates (Figure 10), suggesting that the association of polyphenols with KEMs is a surface phenomenon.

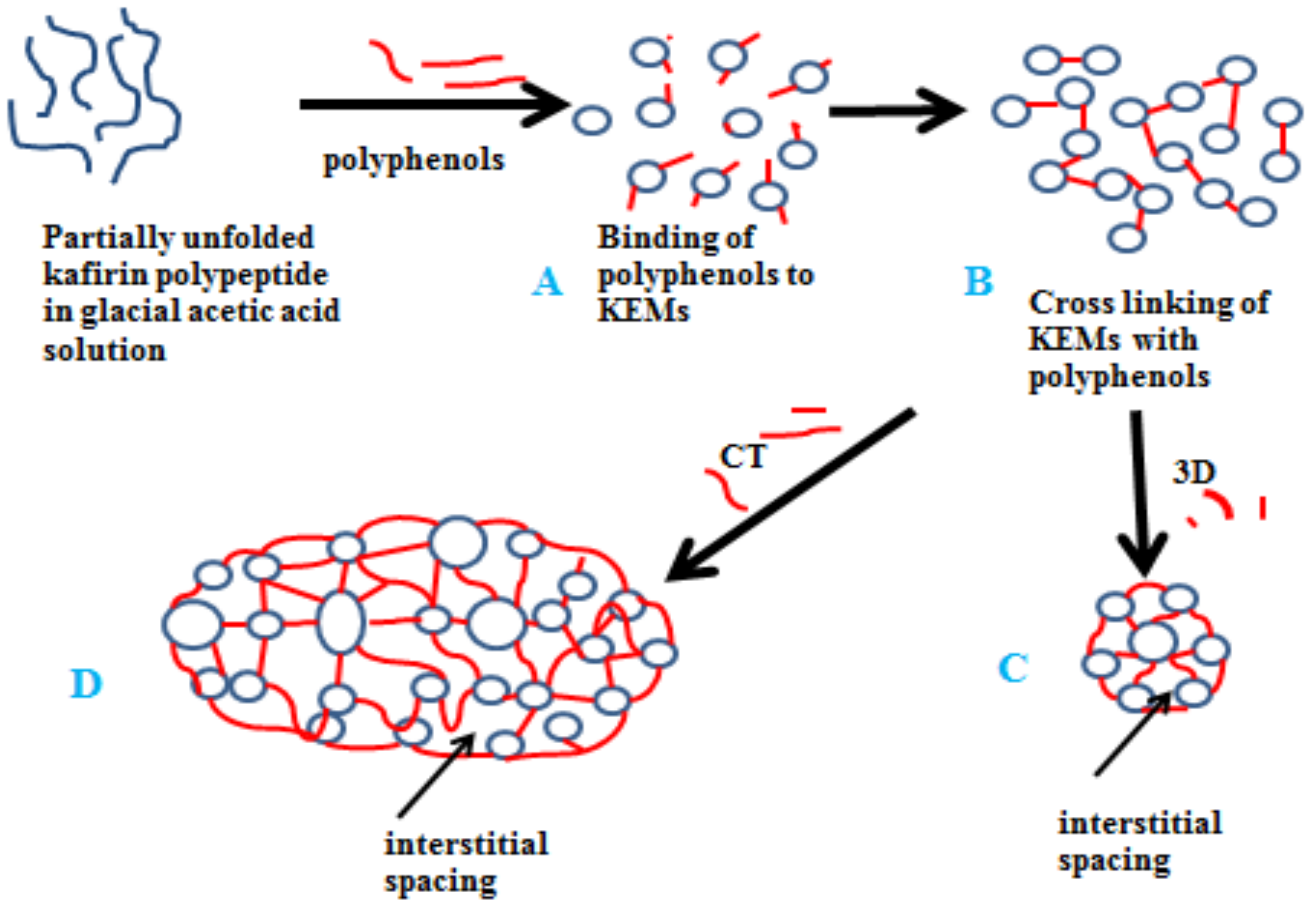


Figure 10: Proposed model for the cross-linking of kafirin with polyphenols (CT: condensed tannins, 3D:3-deoxyanthocyanins) to form KEMs. **A**- Binding of polyphenols on the surface of KEMs **B**- upon addition of more polyphenol, inter-molecular cross linking of KEMs by polyphenols occurs, **C** and **D**- once the cross linked KEMs are stabilized, they precipitate as large aggregates of KEMs

In the hypothetical model proposed by Taylor (2008) to describe the formation of KEMs, it was suggested that the hexapeptide repeat of γ-kafirin would use a self-assembly mechanism to coat the surface of the formed KEMs and thus stabilize the KEMs by disulphide bonds. The hexapeptide repeat (Pro, Pro, Pro, Val, His, Lys) in kafirin is rich in proline (Belton et al., 2006) (Chapter 2.3.2; Table 1). Also as the KEMs contain the same kafirin classes as the original kafirin (Taylor, 2008), it may be assumed that the binding of polyphenols to KEMs is through hydrogen bonding and hydrophobic interactions. The polyphenols will seek out preferred regions on the KEMs surface where its aromatic rings are readily accommodated by the formation of hydrophobic interactions. And this stage, due to the high binding affinity of polyphenols, in particular CT, hydrogen bonding between the phenolic residues and the polar groups of the KEMs will also form, accompanied by the cross-linking of adjacent KEMs with polyphenols. Once a stable cross linked KEMs is formed, with increase in particle size, the KEMs precipitate as large aggregates. This is believed to occur simultaneously.

The resulting cross-linked KEMs were oval-shaped with enlarged vacuole sizes. The oval shape is due to the rate of coalescence being greater than the rate of collision (Lehtinen and Zachariah (2001). The vacuoles in the cross-linked KEMs are thought to be footprints of air bubbles incorporated in the viscous kafirin solution, which become entrapped within the microparticles as they form (Taylor et al., 2009a). As the large cross-linked KEMs appear to be numerous KEMs cross-linked together, the numerous vacuoles which are enlarged in some parts of the KEMs seem to be as a result of interstitial spacing during cross-linking, resulting from the dominant repulsive forces present in the system. Anyango (2012) noted differences in vacuole size between KEMs cross-linked with heat and with glutaraldehyde to be due to either repulsive or attractive forces. Farrer and Lips (1999) put forward a model of below and above close packing of casein micelles formed from sodium caseinate. These authors described below close packing to be dominated by attractive interactions between submicelles and repulsive forces of micellar crowding in above close packing. Thus, it could be expected that the cross-linked KEMs would be dominated by repulsive forces, which could eventually result in larger microparticles being formed with bigger vacuoles (which could also be interstitial spacing between the KEMs) as shown in Figure 10.

The difference in particle size between CT and 3-deoxyanthocyanins cross-linked KEMs can be attributed to their relative size, molecular weight and binding affinity to kafirin. According to Baxteret al. (1997), CT possesses an abundance of phenolic groups, having molecular weight in the range of 500-5000 Da. However, low-molecular weight flavonoids such as 3-deoxyanthocyanins have low affinity to complex kafirin, as was demonstrated by Emmambux and Taylor (2003) and far less phenolic groups than CT (Chapter 2.2.1). The binding was only observed when high concentrations of the non-tannin polyphenols were used. Thus, the 3-deoxyanthocyanins used to cross-link kafirin during microparticle formation resulted in a change in morphology, but not as substantial as with CT. This agrees with Spencer, Cai, Martin, Gaffney, Goulding, Magnolato, Lilley and Haslam (1988), who suggested that simple phenols are able to precipitate proteins from solution when present in high concentrations through the formation of hydrophobic interactions on the surface of the protein.

6.3 Future research and potential applications

For future research, different tannins can be used as cross-linking agents as they possess wide structural diversity (Rice-Evans, Miller and Paganga., 1996). Since sorghum bran phenolic extracts were used in this study without further purification, pure commercial tannins would have fewer or no impurities, thus shedding more light on the interaction of tannins with kafirin and possibly resulting in even sized particles. Also, tannins extracted from different sources would be used to manipulate the morphology and antioxidant properties of microparticles, for example use of grape juice and coffee (Strauss and Gibson, 2004). Lastly, future research will be needed to understand the formation of KEMs and their cross-linking behavior on a molecular level to gain more insight on the processes involved and thus manipulate the functional properties more effectively for specific need use processes (Taylor, 2008).

Potential uses of protein microparticles were highlighted in Chapter 2.1.5. Emmambux, Stading and Taylor (2004) showed that cross-linking kafirin using CT improved the kafirin films in terms of biodegradation, decreased protein digestibility and oxygen permeability with an improvement in the tensile stress. Taylor et al. (2009b) reported reduced protein digestibility of KEMs encapsulated with sorghum condensed tannins. Also Anyango et al.

(2012), cross-linked KEMs with heat and glutaraldehyde treatments, and reported an improvement in the functional properties of cross-linked KEMs such as entrapment efficiency with an increase in particle size. Cross-linking KEMs with polyphenols prepared in this study could be expected to have improved functional properties such as loading capacity, improved mechanical strength and less prone to enzymatic attack, enhancing sustained delivery. Thus, in potential application, encapsulation of other bioactive compounds, such as β-carotene (pro-vitamin A) should be investigated.

Development of biomaterials for tissue engineering and drug delivery for medical applications could be another potential use of the cross-linked KEMs. According to Reddy and Yang (2011), protein microparticles are preferred because proteins are a major part of the human body; are bio- and cyto-compatible.

7 CONCLUSION AND RECOMMENDATIONS

This study has established that the morphology of KEMs can be manipulated by cross-linking kafirin with polyphenols to microparticle preparation by coacervation in a simple novel single step process. By varying the type and amount of polyphenol (CT and 3-deoxyanthocyanins) used during coacervation, the characteristics of the microparticles could be manipulated to a desired structure and size as shown in the proposed model (Figure 10). Condensed tannins have high molecular weights and multiple binding sites compared to 3-deoxyanthocyanins, thus they can cross-link KEMs at more sites until stable complexes are formed. As more polyphenol is added, binding sites for KEMs increases resulting also in an increase in the average diameter of the KEMs. As a result of the cross-linking, more interstitial spaces are formed, that can entrap more air giving rise to highly porous cross-linked KEMs, with enlarged vacuole sizes.

The resulting highly porous large cross-linked KEMs would have improved functional properties. For example, the cross-linked KEMs would possibly encapsulate more active ingredients due to their large size and release them over long periods of time. Also, access of digestive enzymes and release of encapsulated ingredients is enabled due to their porous nature. Since KEMs are hydrophobic-based systems, the KEMs are capable of achieving sustained ingredient release because of their stability in aqueous environments and low susceptibility to human enzymes. However, KEMs cross-linked with condensed tannins not only resulted in large oval shaped KEMs with increased porosity but also contributed significantly to the antioxidative benefits of the resultant KEMs. The additional antioxidative benefit of the KEMs cross-linked with CT would also be available to act as a free radical sink within the gastrointestinal tract. Hence, cross-linking of kafirin during coacervation with sorghum polyphenols result in shape change with increased particle size and porosity, which may have potential application in the food and biomedical industry as encapsulation agents with improved performance. However, future work is needed to exploit the cross-linked KEMs by encapsulating with active ingredients, for example, β-carotene in achieving sustained release rates and also as biomaterials for tissue engineering and drug delivery.

8 REFERENCES

Akin, H., Hasirci, N., 1995. Preparation and characterization of cross-linked gelatin microspheres. Journal of Applied Polymer Science 58, 95-100.

Allèmann, E., Leroux, J., Gurny, R., 1998. Polymeric nano- and microparticles for the oral delivery of peptides and peptidomimetics. Advanced Drug Delivery Reviews 34, 171–189.

American Association of Cereal Chemists (AACC), 2000. Crude protein-combustion, Standard Method 46-30. Approved Methods of the AACC (10th edition). The Association, St Paul, MN.

Anyango, J.O., 2012. Physico-chemical modification of kafirin microstructures for application as biomaterials. PhD Thesis. University of Pretoria, Pretoria.

Anyango, J.O., Duneas, N., Taylor, J.R.N., Taylor, J., 2012. Physicochemical modification of kafirin microparticles and their ability to bind bone morphogenetic protein‐2 (BMP-2), for application as a biomaterial. Journal of Agricultural and Food Chemistry 60, 8419−8426.

Arshady, R., 1990. Microspheres and microcapsules, a survey of manufacturing techniques Part II: Coacervation. Polymer Engineering and Science 30, 905-914.

Augustin, M.A., Hemar, Y., 2009. Nano- and micro-structured assemblies for encapsulation of food ingredients. Chemical Society Reviews 38, 902-912.

Awika, J.M., Rooney, L.W., 2004. Sorghum phytochemicals and their potential impact on human health. Phytochemistry 65, 1199-1221.

Awika, J.M., Rooney, L.W., Waniska, R.D., 2004a. Anthocyanins from black sorghum and their antioxidant properties. Food Chemistry 90, 293-301.

Awika, J.M., Rooney, L.W., Waniska, R.D., 2004b. Properties of 3-deoxyanthocyanins from sorghum. Journal of Agricultural and Food Chemistry 52, 4388-4394.

Awika, J.M., Rooney, L.W., Wu, X., Prior, R.L., Zevallos, L.C., 2003. Sceening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. Journal of Agricultural and Food Chemistry 51, 6657-6662.

Baxter, N.J., Lilley, T.H., Haslam, E., Williamson, M.P., 1997. Multiple interactions between polyphenols and a salivary proline-rich protein repeat result in complexation and precipitation. Biochemistry 36, 5566-5577.

Beaulieu, L., Savoie, L., Paquin, P., Subirade, M., 2002. Elaboration and characterization of whey protein beads by an emulsification/cold gelation process: application for the protection of retinol. Biomacromolecules 3, 239–248.

Belton, P.S., Delgadillo, I., Halford, N.G., Shewry, P.R., 2006. Kafirin structure and functionality. Journal of Cereal Science 44, 272–286.

Benshitrit, R.C., Levi, C.S., Tal, S.L., Shimoni, E., Lesmes, U., 2012. Development of oral food-grade delivery systems: Current knowledge and future challenges. Food and Function 3, 10-21.

Betz, M., Kulozik, U., 2011. Whey protein gels for the entrapment of bioactive anthocyanins from bilberry extract. International Dairy Journal 21, 703-710.

Bravo, L., 1998. Polyphenols: Chemistry, dietary sources, metabolism and nutritional significance. Nutrition Reviews 56, 317-333.

Burgess, D.J., Hickey, A.J., 1994. Microsphere technology and applications. In: Swarbrick, J., Boylan, J.C (Eds), Encyclopedia of Pharmaceutical Technology, Marcel Dekker, New York, Volume 10, pp. 1-29.

Butler, L.G., 1982. Relative degree of polymerization of sorghum tannin during seed development and maturation. Journal of Agricultural and Food Chemistry 30, 1090-1094.

Butler, L.G., Riedl, D.J., Lebryk, D.G., Blytt, H.J. 1984. Interaction of proteins with sorghum tannins. Mechanism, specificity and significance. Journal of the American Oil Chemists Society 6, 916-920.

Calderon, P., Van Buren, J., Willard, B.R., 1968. Factors influencing the formation of precipitates and hazes by gelatin and condensed and hydrolysable tannins. Journal of Agricultural and Food Chemistry 16, 479-482.

Casuccio, G.S., Schlaegle, S.F., Lersch, T.L., Huffman, G.P., Chen, Y., Shah, N., 2004. Measurement of fine particulate matter using electron microscopy techniques. Fuel Processing Technology 85, 763– 779.

Charlton, A.J., Baxter, N.J., Khan, M.L., Moir, A.J.G., Haslam, E., Davies, A.P., Williamson, M.P., 2002. Polyphenol/peptide binding and precipitation. Journal of Agriculture 50, 1593-1601.

Chen, L., Remondetto, G.E., Subirade, M., 2006. Food protein-based materials as nutraceutical delivery systems. Trends in Food Science and Technology 17, 272–283.

Chen, L., Subirade, M., 2007. Effect of preparation conditions on the nutrient release properties of alginate-whey protein granular microspheres. European Journal of Pharmaceutics and Biopharmaceutics 65, 354–362.

Chen, L., Subirade, M., 2009. Elaboration and characterization of soy/zein protein microspheres for controlled nutraceutical delivery. Biomacromolecules 10, 3327–3334.

Conklin, K.A., 2000. Dietary antioxidants during cancer chemotherapy: Impact on chemotherapeutic effectiveness and development of side effects. Nutrition and Cancer 37, 1–18.

Cooper, C., Corredig, M., Alexander, M., 2010. Investigation of the colloidal interactions at play in combined acidification and rennet of different heat-treated milks. Journal of Agricultural and Food Chemistry, 58, 4915-4922.

Cornacchia, L., Roos, Y.H., 2011. Stability of β-carotene in protein-stabilized oil-in-water delivery systems. Journal of Agricultural and Food Chemistry 59, 7013-7020.

Daiber, K.H., Taylor, J.R.N., 1982. Effects of formaldehyde on protein extraction and quality of high- and low-tannin sorghum. Journal of Agricultural and Food Chemistry 30, 70-72.

Damink, L.H.H.O., Dijkstra, P.J., van Luyn, M.J.A., van Wachem, P.B., Nieuwenhuis, P., Feijen, J., 1995. Gluteraldehyde as a cross-linking agent for collagen-based biomaterials. Journal of Materials Science: Materials in Medicine 6, 460-472.

Das, L., Bhaumik, E., Raychaudhuri, U., Chakraborty, R., 2011. Role of nutraceuticals in human health. Journal of Food Science and Technology 49, 173-183.

De Jong, G.A.H., Koppelman, S.J., 2002. Transglutaminase catalyzed reactions: Impact on food applications. Journal of Food Science 67, 2798-2806.

De Vos, P., Faas, M.M., Spasojevic, M., Sikkema, J., 2010. Encapsulation for preservation of functionality and targeted delivery of bioactive food components. International Dairy Journal 20, 292–302.

DeRose, R., Ma, D.P., Kwon, I.S., Hasnain, S.E., Klassy, R.C., Hall, T., 1989. Characterization of the kafirin gene family reveals extensive homology with zein from maize. Plant Molecular Biology 12, 245-256.

Desai, K.G.H., Park, H.J., 2005. Recent developments in microencapsulation of food ingredients. Drying Technology 23, 1361–1394.

Dubey, R., Shami, T.C., Rao, K.U.B., 2009. Microencapsulation technology and applications. Defence Science Journal 59, 82-95.

Duodu, K.G., Taylor, J.R.N., Belton, P.S., Hamaker, B.R., 2003. Factors affecting sorghum protein digestibility. Journal of Cereal Science, 38, 117-131.

Dykes, L., Rooney, L.W., 2006. Sorghum and millet phenols and antioxidants. Journal of Cereal Science 44, 236-251.

Dykes, L., Rooney, L.W., 2007. Phenolic compounds in cereals. Cereal Foods World 52, 105-111.

Dykes, L., Rooney, L.W., Waniska, R.D., Rooney, W.L., 2005. Phenolic compounds and antioxidant activity of sorghum grains of varying genotypes. Journal of Agricultural and Food Chemistry 53, 6813-6818.

Dykes, L., Seitz, L.M., Rooney, W.L., Rooney, L.W., 2009. Flavonoid composition of red sorghum genotypes. Food Chemistry 116, 313–317.

El Nour, I.N.A., Peruffo, A.D.B., Curioni, A., 1998. Characteristics of sorghum kafirins in relation to their cross-linking behaviour. Journal of Cereal Science 28, 197-207.

Elzoghby, A.O., Samy, W.N., Elgindy, N.A., 2012. Protein-based nanocarriers as promising drug and gene delivery systems. Journal of Controlled Release 161, 38–49.

Emmambux, M.N., Stading, M., Taylor, J.R.N., 2004. Sorghum kafirin film property modification with hydrolysable and condensed tannins. Journal of Cereal Science 40, 127–135.

Emmambux, N.M., Taylor, J.R.N., 2003. Sorghum kafirin interaction with various phenolic compounds. Journal of the Science of Food and Agriculture 83, 402-407.

Esposito, E., Cortesi, R., Nastruzzi, C., 1996. Gelatin microspheres: influence of preparation parameters and thermal treatment on chemico-physical biopharmaceutical properties. Biomaterials 17, 2009-2020.

Evans, D.J., Schüssler, L., Taylor, J.R.N., 1987. Isolation of reduced-soluble protein from sorghum starchy endosperm. Journal of Cereal Science 5, 61-65.

Fang, Z., Bhandari, B., 2010. Encapsulation of polyphenols- a review. Trends in Food Science and Technology 21, 510-523.

Farrer, D., Lips, A., 1999. On the self-assembly of sodium caseinate. International Dairy Journal 9, 281-286.

Fernandez, A., Torres-Giner, S., Lagaron, J.M., 2009. Novel route to stabilization of bioactive antioxidants by encapsulation in electrospun fibers of zein prolamin. Food Hydrocolloids 23, 1427–1432.

Frazier, R.A., Deaville, E.R., Green, R.J., Stringano, E., Willoughby, I., Plant, J., Mueller-Harvey, I., 2010. Interactions of tea tannins and condensed tannins with proteins. Journal of Pharmaceutical and Biomedical Analysis 51, 490–495.

Freitas, S., Merkle, H.P., Gander, B., 2005. Microencapsulation by solvent extraction/evaporation: Reviewing the state of the art of microsphere preparation process technology. Journal of Controlled Release 102, 313–332.

Gao, C., Taylor, J., Wellner, N., Byaruhanga, Y.B., Parker, M.L, Mills, E.N.C., Belton, P.S., 2005. Effect of preparation conditions on protein secondary structure and biofilm formation of kafirin. Journal of Agricultural and Food Chemistry 53, 306-312.

Gerrard, J.A., 2002. Protein–protein crosslinking in food: Methods, consequences, applications. Trends in Food Science and Technology 13, 391–399.

Gharsallaoui, A., Roudaut, G., Chambin, O., Voilley, A., Saurel, R., 2007. Applications of spray-drying in microencapsulation of food ingredients: An overview. Food Research International 40, 1107−1121.

Gómez-Guillén, M.C., Giménez, B., López-Caballero, M.E., Montero, M.P., 2011. Functional and bioactive properties of collagen and gelatin from alternative sources: A review. Food Hydrocolloids 25, 1813-1827.

Gunasekaran, S., Ko, S., Xiao, L., 2007. Use of whey proteins for encapsulation and controlled delivery applications. Journal of Food Engineering 83, 31–40.

Hagerman, A.E., Butler, L.G., 1981. The Specificity of Proanthocyanidin-Protein Interactions. The Journal of Biological Chemistry 256, 4494-4497.

Hagerman, A.E., Rice, M.E., Ritchard, N.T., 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin (4-8) catechin (procyanidin). Journal of Agricultural and Food Chemistry 46, 2590-2595.

Hahn, D. H., Rooney, L. W., Earp, C. F., 1984. Tannins and phenols of sorghum. Cereal Foods World 29, 776-779.

Health Canada., 1998. Policy paper. Therapeutic products programme and the food directorate from the health protection branch. Nutraceuticals/ functional foods and health claims on foods. Health Canada, Ottawa, pp. 1-29. http://www.hc-sc.gc.ca/fn-an/alt_formats/hpfb-dgpsa/pdf/label-etiquet/nutra-funct_foods-nutra-fonct_aliment-eng.pdf (accessed 15 January 2013).

Heidebach, T., Först, P., Kulozik, U., 2009. Transglutaminase-induced caseinate gelation for the microencapsulation of probiotic cells. International Dairy Journal 19, 77–84.

Hennink, W.E., van Nostrum, C.F., 2012. Novel crosslinking methods to design hydrogels. Advanced Drug Delivery Reviews 64, 223-236.

Herrmann, J., Bodmeier, R., 1998. Biodegradable, somatostatin acetate containing microspheres prepared by various aqueous and non-aqueous solvent evaporation methods. European Journal of Pharmaceutics and Biopharmaceutics 45, 75–82.

Hoffman, A.S., 2002. Hydrogels for biomedical applications. Advanced Drug Delivery Reviews 43, 3–12.

Jenkins, J.A., Breiteneder, H., Mills, E.N., 2007. Evolutionary distance from human homologs reflects allergenicity of animal food proteins. Journal of Allergy and Clinical Immunology 120, 1399-1405.

Jöbstl, E., O'Connell, J., Fairclough, P.A., Williamson, M.P., 2004. Molecular model for astringency produced by polyphenol/protein interactions. Biomacromolecules 5, 942-949.

Jones, O.W., McClements, D.J., 2010. Functional biopolymers particles: Design, fabrication and applications. Comprehensive Reviews in Food Science and Food Safety 9, 374-397.

Kalia, AN., 2005. Textbook of Industrial Pharmacology, CBS Publishers and Distributors, New Delhi, pp 204–208.

Kim, S., Xu, J., 2008. Aggregate formation of zein and its structural inversion in aqueous ethanol. Journal of Cereal Science 47, 1–5.

Korus, J., 2001. Microencapsulation of flavors in starch matrix by coacervation. Polish Journal of Food Nutritional Science, 10, 17–23.

Lai, L.F., Guo, H.X., 2011. Preparation of new 5-fluorouracil-loaded zein nanoparticles for liver targeting. International Journal of Pharmacy 404, 317–323.

Lambert, J.D., Hong, J.H., Yang, G., Liao, J., Yang, C., 2005. Inhibition of carcinogenesis by polyphenols: Evidence from laboratory investigations. The American Journal of Clinical Nutrition 81, 284-291.

Latha, M.S., Jayakrishman, A., 1994. Glutaraldehyde cross-linked bovine casein microspheres as a matrix for the controlled release of theophylline: In-vitro studies. Journal of Pharmacy and Pharmacology 46, 8-13.

Lee, S.J., Rosenberg, M., 2000. Microencapsulation of theophylline in whey proteins: Effects of core-to-wall ratio. International Journal of Pharmaceuticals 205, 147-158.

Lehtinen, K.E.J., Zachariah, M.R., 2001. Effect of coalescence energy release on the temporal shape evolution of nanoparticles. Physical Review B 63, 205402-1–205402-7.

Li, Y., Li, J., Xia, Q., Zhang, B., Wang, Q., Huang, Q., 2012. Understanding the dissolution of α-zein in aqueous ethanol and acetic acid solutions. The Journal of Physical Chemistry 116, 12057-12064.

Liu, X., Sun, Q., Wang, H., Zhang, L., Wang, J.Y., 2005. Microspheres of corn protein, zein, for an ivermectin drug delivery system. Biomaterials 26, 109–115.

Liyana-Pathirana, C.M., Shahidi, F., 2005. Antioxidant activity of commercial soft and hard wheat (*Triticum aestivum L.*) as affected by gastric pH conditions. Journal of Agricultural and Food Chemistry 53, 2433-2440.

Loveday, S.M., Singh, H., 2008. Recent advances in technologies for vitamin A protection in foods. Trends in Food Science and Technology 19, 657-668.

MacAdam, A.B., Shaft, Z.B., James, S.L., Marriott, C., Martin, G.P., 1997. Preparation of hydrophobic and hydrophilic albumin microspheres and determination of surface carboxylic acid and amino residues. International Journal of Pharmaceutics 151, 47 55.

MacDonald-Wicks, L.K., Wood, L.G., Garg, M.L., 2006. Methodology for the determination of biological antioxidant capacity *in vitro*: A review. Journal of the Science of Food and Agriculture 86, 2046–2056.

Madene, A., Jacquot, M., Scher, J., Desobry, S., 2006. Flavour encapsulation and controlled release – A review. International Journal of Food Science and Technology 41, 1–21.

Mahler, H.C., Freiess, W., Grauschopf, U., Kiess, S., 2009. Protein aggregation: Pathways, induction factors and analysis. Journal of Pharmaceutical Sciences 98, 2909-2934.

Marchand, L.L., 2002. Cancer preventive effects of flavonoids- A review. Biomedicine and Pharmacotherapy 56, 296-301.

Matalanis, A., Jones, Q.G., McClements, D.J., 2011. Structured biopolymer-based delivery systems for encapsulation, protection and release of lipophilic compounds. Food Hydrocolloids 25, 1865-1880.

Mazhar, H., Chandrashekar, A., Shetty, H.S., 1993. Isolation and immumochemical characterization of the alcohol-soluble proteins (kafirins) of Sorghum bicolor (L.) Moench. Journal of Cereal Science 17, 83-93.

McManus, J.P., Davis, K.G., Beart, J.E., Gaffney, S.H., Lilley, T.H., Haslam, E., 1985. Polyphenol interactions. Part 1. Introduction; some observations on the reversible complexation of polyphenols with proteins and polysaccharides. Journal of Chemistry Society 11, 1429-1438.

Mesa-Sonestreet, N.J., Alavi, S., Bean, S.R., 2010. Sorghum proteins: the concentration, isolation, modification and food applications of kafirins. Journal of Food Science 75, 90-104.

Murugesan, R., Orsat, V., 2012. Spray drying for the production of nutraceutical ingredients-A review. Food Bioprocess Technology 5, 3-14.

Naczk, M., Shahidi, F., 2004. Extraction and analysis of phenolics in foods. Journal of Chromatography 1054, 95–111.

Nesterenko, A., Alric, I., Silvestre, F., Durrieu, V., 2013. Vegetable proteins in microencapsulation: A review of recent interventions and their effectiveness. Industrial Crops and Products 42, 469-479.

Nicolai, T., Britten, B., Schmitt, S., 2011. β-Lactoglobulin and WPI aggregates: Formation, structure and applications. Food Hydrocolloids 25, 1945-1962.

Norton, I.T., Frith, W.J., 2001. Microstructure design in mixed biopolymer composites. Food Hydrocolloids 15, 543-553.

Patil, G.V., 2003. Biopolymer albumin for diagnosis and in drug delivery. Drug Development Research 58, 219–247.

Peppas, N.A., Buresa, P., Leobandung, W., Ichikawa, H., 2000. Hydrogels in pharmaceutical formulations. European Journal of Pharmaceutics and Biopharmaceutics 50, 27-46.

Pèrez-Jimènez, J., Saura-Calixto, F., 2006. Effect of solvent and certain food constituents on different antioxidant capacity assays. Food Research International 39, 791–800.

Picot, A., Lacroix, C., 2004. Encapsulation of bifidobacteria in whey protein-based microcapsules and survival in simulated gastrointestinal conditions and in yoghurt. International Dairy Journal 14, 505–515.

Prakash, D., Gupta, K.R., 2009. The antioxidant phytochemicals of nutraceutical importance. The Open Nutraceuticals Journal 2, 20-35.

Prior, R.L., Wu, X., Schaich, K., 2005. Standardized methods for the determination of antioxidant capacity and phenolics in foods and dietary supplements. Journal of Agricultural and Food Chemistry 53, 4290-4302.

Reddy, N., Yang, Y., 2011. Potential of plant proteins for medical applications. Trends in Biotechnology 29, 490-498.

Reichert, R.D., Tyler, R.T., York, A.E., Schwab, D.J., Tatarynovich, J.E., Mwasaru, M.A., 1986. Description of a production model of the tangential abrasive dehulling device and its application to breeder' samples. Cereal Chemistry 63, 201-207.

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 20, 933-956.

Rice-Evans, C.A., Miller, N.J., Paganga, G., 1997. Antioxidant properties of phenolic compounds. Trends in Plant Science 2, 152-159.

Riedl, K.M., Hagerman, A.E., 2001. Tannin-protein complexes as radical scavengers and radical sinks. Journal of Agricultural and Food Chemistry 49, 4917-4923.

Risch, S.J., 1995. Encapsulation: Overview of uses and techniques. American Chemical Society (Eds), pp 1-7.

Robbins, R.J., 2003. Phenolic acids in foods: An overview of analytical methodology. Journal of Agricultural and Food Chemistry 51, 2866−2887.

Robbins, R.J., Kwik-Uribe, C., Hammerstone, J.F, Schmitz, H.H., 2006. Analysis of flavanols in foods: What methods are required to enable meaningful health recommendations? Journal of Cardiovascular Pharmacology 47, S110-S118.

Scalbert, A., Johnson, I.T., Saltmarsh, M., 2005a. Polyphenols: Antioxidants and beyond. American Journal of Clinical Nutrition 81, 215S–217S.

Scalbert, A., Manach, C., Morand, C., Rémésy, C., Jimènez, L., 2005b. Dietary polyphenols and the prevention of diseases. Critical Reviews in Food Science and Nutrition 45, 287–306.

Shahidi, F., Han, X.Q., 1993. Encapsulation of food ingredients. Critical Reviews in Food Science and Nutrition 33, 501-547.

Shewry, P.R., 2002. The major seed storage proteins of spelt wheat, sorghum, millets and pseudocreals. In: Belton, P.S., Taylor, J.R.N, (Eds), Pseudocereals and Less Common Cereals, Springer, Berlin, pp. 1-24.

Shull, J.M., Watterson, J.J., Kirleis, A.W., 1991. Proposed nomenclature for the alcohol-soluble proteins (kafirins) of Sorghum bicolor (L. Moench) based on molecular weight, solubility and structure. Journal of Agricultural and Food Chemistry 39, 83–87.

Siebert, K.J., Troukhanova, N.V., Lynn, P.Y., 1996. Nature of polyphenol-protein interactions. Journal of Agricultural and Food Chemistry 44, 80-85.

Singleton, V.L., Rossi, J., 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. American Journal of Enology and Viticulture 16, 144–158.

Sinha, V.R., Trehan, A., 2003. Biodegradable microspheres for protein delivery. Journal of Controlled Release 90, 261–280.

Spencer, C.M., Cai, Y., Martin, R., Gaffney, S.H., Goulding, P.N., Magnolato, D., Lilley, T.H., Haslam, E., 1988. Polyphenol complexation: Some thoughts and observation. Phytochemistry 27, 2397-2409.

Stadtländer, C.T.K.H., 2007. Scanning electron microscopy and transmission electron microscopy of mollicutes: challenges and opportunities. In Mendez-Vilas, A., Diaz, J., (Eds), Modern Research and Educational Topics in Microscopy, St Paul, MN, USA, pp. 122-131.

Stadtländer, C.T.K.H., Kirchhoffa, H., 2004. Balanced technique for preparation of specimens from pathogenicity studies for scanning electron microscopy. Scanning 26, 175–180.

Strauss, G., Gibson, S.M., 2004. Plant phenolics as cross-linkers of gelatin gels and gelatin-based coacervates for use as food ingredients. Food Hydrocolloids 18, 81-89.

Taylor, J., 2008. Preparation, characterisation and functionality of kafirin microparticles. PhD Thesis. University of Pretoria, Pretoria.

Taylor, J., Bean, S.R., Ioerger, B.P., Taylor, J.R.N., 2007. Preferential binding of sorghum tannins with γ-kafirin and the influence of tannin binding on kafirin digestibility and biodegradation. Cereal Chemistry 46, 22-31.

Taylor, J., Taylor, J.R.N., Belton, P.S., Minnaar, A., 2009a. Formation of kafirin microparticles by phase separation from an organic acid and their characterisation. Journal of Cereal Science 50, 99-105.

Taylor, J., Taylor, J.R.N., Belton, P.S., Minnaar, A., 2009b. Kafirin microparticle encapsulation of catechin and sorghum condensed tannins. Journal of Agricultural and Food Chemistry 57, 7523-7528.

Taylor, J.R.N., Belton, P.S., 2002. Sorghum. In: Belton, P.S., Taylor, J.R.N., (Eds), Pseudocereals and Less Common Cereals, Berlin, Springer, pp. 25-38.

Tripoli, E., Guardia, M.L., Giammanco, S., Majo, D.D., Giammanco, M., 2007. Citrus flavonoids: Molecular structure, biological activity and nutritional properties: A review. Food Chemistry 104, 466-479.

Van der Linden, E., Venema, P., 2007. Self-assembly and aggregation of proteins. Current Opinion in Colloid and Interface Science 12, 158–165.

Vandelli, M.A., Rivasi, F., Guerra, P., Forni, F., Arletti, R., 2001. Gelatin microspheres cross-linked with D,L-glyceraldehyde as a potential drug delivery system: Preparation, characterization, in vitro and in vivo studies. International Journal of Pharmaceutics 215, 175–184.

Vandelli, M.A., Romagnoli, M., Monti, A., Gozzi, M., Guerra, P., Rivasi, F., Forni, F., 2004. Microwave-treated gelatin microspheres as drug delivery system. Journal of Controlled Release 96, 67–84.

Watterson, J.J., Shull, J.M., Kirleis, A.W., 1993. Quantification of α-, β- and γ-kafirins in vitreous and opaque endosperm of Sorghum bicolor. Cereal Chemistry 70, 452-457.

Waxman, A., Norum, K.R., 2004. Why a global strategy on diet, physical activity and health? The growing burden of non-communicable diseases. Public Health Nutrition 7, 381-383.

Whitesides, G.M., Mathias, J.P., Seto, C., 1991. Molecular, self-assembly and nanotechnology: A chemical strategy for synthesis of nanostructures. Science 254, 1312–1319.

Wilson, N., Shah, N.P., 2007. Microencapsulation of vitamins. ASEAN Food Journal 14, 1-14.

Wong, S.P., Leong, L.P., Koh, J.H.W., 2006. Antioxidant activities of aqueous extracts of selected plants. Food Chemistry 99, 775–783.

Yang, H., Wang, Y., Lai, S., An, H., Li, Y., Chen, F., 2007. Application of atomic force microscopy as a nanotechnology tool in food science. Journal of Food Science 72, 65-75.

Zhang, S., 2002. Emerging biological materials through molecular self-assembly. Biotechnology Advances 20, 321–339.

Zhang, W., Zhong, Q., 2009. Microemulsions as nanoreactors to produce whey protein nanoparticles with enhanced heat stability by sequential enzymatic cross-linking and thermal pretreatments. Journal of Agricultural and Food Chemistry 57, 9181–9189.

Zhang, X., Do, M.D., Casey, P., Sulistio, A., Qiao, G.G., Lundin, L., Lillford, P., Kosaraju, S., 2010. Chemical modification of gelatin by a natural phenolic cross-linker, tannic acid. Journal of Agricultural and Food Chemistry 58, 6809–6815.

Zhao, H., Gagnon, J., Häfeli, U.O., 2007. Process and formulation variables in the preparation of injectable and biodegradable magnetic microspheres. BioMagnetic Research and Technology 5, 1-11.