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Stearic acid addition to tef and maize starches and its influence on their functional properties

by

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Declaration

I declare that this dissertation submitted at the University of Pretoria for the degree MSc Food Science has not been submitted by me for a degree at any other University or Institution of higher education

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ABSTRACT

Starch is applied in food products as a thickener, gelling agent and fat replacer. The modification of native starch using a naturally derived chemical like stearic acid could bring about improved starch properties, for example reduced retrogradation and stronger gels, without the risk associated with the use of synthetic chemicals. Tef is considered to be underutilized and under-researched. This project investigated the microstructure of tef and maize starch pastes as affected by stearic acid addition using light microscopy, scanning electron microscopy (SEM) and X-ray diffraction. The thermal properties of tef and maize starches were also investigated with Differential Scanning Calorimetry (DSC).

Starch suspensions (10% w/v) containing stearic acid (0.25% and 1.50%) were subjected pasting with an extended holding period of 2 or 4 hours at 91 °C in a Rapid Visco Analyser (RVA). As expected, a large second viscosity peak was observed for both tef and maize starches modified with stearic acid. This second pasting peak increased in size with increased concentration of stearic acid. No second viscosity peak was observed with tef control and only a small second peak was observed with maize control. This suggests that the formation of amylose-stearic acid complexes was responsible for the second peak formation. Additionally, tef starch modified with 0.25% stearic acid did not reach a first peak viscosity and increased in viscosity throughout holding, while for 1.50% stearic acid there was decreased peak viscosity and increased time to peak viscosity.

At predetermined points during pasting of tef and maize starches (controls and modified with stearic acid), starch paste samples were taken and immediately plunge frozen in liquid nitrogen (-180°C). The freshly thawed samples were analysed using light microscopy. For SEM, X-ray diffraction and DSC analysis, frozen samples were freeze dried. Light microcopy and SEM showed that both tef and maize starch pastes contained large proportions of intact granules up to second peak formation. This was suggested to be due to the interaction of stearic acid with the starch granules. A few crystalline structures (1-10µm) were

observed for maize starch modified with 1.50% stearic acid and sampled at the second peak. However, there was not sufficient microscopical evidence suggesting the presence of micron sized crystalline amylose-stearic acid complexes. X-Ray diffraction supported the presence of amylose-stearic acid complexes because of the formation of 0.44 nm, 0.68 nm and 1.2 nm peaks These were observed for both tef and maize starch pastes sampled at the first viscosity peak, the second viscosity peak and at the end of pasting. The size of the X-ray diffraction peaks, however, was largest for tef and maize starch (modified with 1.50% stearic acid) pastes sampled at the second pasting peak. DSC showed endothermic changes in heat flow for tef and maize starches modified with 1.50% stearic acid and sampled at the end of pasting at temperatures of 99 – 120 °C, which corresponds to the melting transitions of amylose-lipid complexes.

Both the X-ray Diffraction and DSC data support the hypothesis that amylose-stearic acid complexes have a role to play in the second pasting peak formation of tef and maize starches. While microscopy has not provided evidence for the existence of such complexes, their presence possibly as nano structures cannot be disregarded and should be investigated in future by use of more sensitive techniques such as Atomic Force Microscopy (AFM) and Small Angle X-ray Scattering (SAXS).

The effects of stearic acid on the microstructures and thermal properties of tef and maize starch pastes are attributed to the formation of amylose-stearic acid complexes. These fatty acid modified starches may be useful as fat replacers due to their high viscosities and low tendency to retrograde.

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CHAPTER 1

INTRODUCTION

Starch is a natural biopolymer that plays both important nutritional and functional roles in food. In terms of functionality, starch can be used in food for its thickening, stabilizing and gelling abilities, but the use of native starch in manufactured food products is limited. Low thermal resistance, high tendency towards retrogradation and rubbery weak-bodied gels are some of the limitations of native starch for food use (reviewed by Singh *et al.*, 2007; BeMiller, 2003). Starch is modified in order to overcome these limitations. Starch can be chemically and physically modified to improve its properties such as thickening, binding, stability, mouthfeel, gelling, dispersion or cloud formation (Singh *et al.*, 2007). However, the modifications with synthetically derived chemicals have certain limitations as consumers prefer the use of naturally derived chemicals. Organizations, for example the United States Food and Drug Administration (FDA) sets limits for chemicals used for modified starches (Wurzburg, 2006). Residues of the chemicals used for production of modified starches may not only pose possible health risks but also lead to unacceptable sensory properties. Thus, the use of natural food chemicals, such as fatty acids, to improve starch properties can be advantageous as they also form part of food.

Fatty acids have been used to alter starch functionality. Kaur & Singh (2000), Godet *et al*. (1995) and Zhou *et al.* (2007) found that the functional properties of maize and rice starches were altered with fatty acid modification. The starches showed decreased peak and breakdown viscosity as well as reduced retrogradation, during pasting. Nelles *et al.* (2000), Bajner (2002) & D’ Silva (2009) found a second increase in viscosity during extended pasting of maize and tef starches complexed with stearic acid. Nelles *et al.* (2000) suggested that the second peak viscosity increase in maize starch can be attributed to the formation of starch-lipid inclusion complexes. The effect of fatty acids on starch properties have mainly been focused on maize, rice and wheat. Research on tef starch have only been reported by D' Silva (2009). Moreover, there is no concrete evidence that the second viscosity increase during extended pasting of starches is due to amylose-lipid complexes.

Tef [*Eragrostis tef* (Zucc.) Trotter] is a tropical cereal that is, not exclusively, but significantly cultivated in Ethiopia (National Research Council, 1996). It is also grown for food in countries such as India, Malawi and Kenya (National Research Council, 1996). It is mainly used as forage crop in South-Africa. Tef is considered to be underutilized, like in the case of sorghum and millet (Taylor *et al.*, 2006), under researched and considered a lost crop of Africa. Tef starch shows reduced peak and setback viscosities as well as increased resistance to breakdown, when compared to maize starch (Bultosa, 2003). These unique properties of tef starch may be further exploited by using stearic acid as a natural alternative in altering it functional properties. This may not only increase its application, but also further develop the potential of the products formed.

CHAPTER 2
LITERATURE REVIEW

This review focuses on research of the interaction between starch and lipids resulting in the formation of amylose-lipid complexes and their possible role in the formation of the second viscosity peak during starch pasting.

2.1 Starch: Composition and structure

Starch is a biomaterial made up of two different polysaccharide fractions, amylose and amylopectin biopolymers (Zobel *et al*., 1988a). Starch is found in plants as semi-crystalline structures in granules of which the size (1-100 µm) (Gunaratne & Corke, 2004), shape and composition differ according to the botanical source (Jackson, 2003). Starch acts as a major carbohydrate reserve. Grain tef, a tropical cereal, has a carbohydrate content of approximately 73% (Bultosa, Hall & Taylor, 2002) of which starch makes up the largest proportion. The average starch content of maize is approximately 71% (Watson, 1984).

2.1.1 Starch granule

“Granules are the basic physical structural unit of starch” (Gunaratne & Corke, 2004). In cereals starch granules are located mostly in the endosperm (Jackson, 2003). In order to identify the size and features of starch granules, several microscopic techniques, such as light microscopy and electron microscopy, have been used (Jackson, 2003). Undamaged native starch granules, viewed under polarized light, exhibit birefringence (Maltese crosses) which confirms a high degree of molecular order (Kent & Evers, 1994).

Tef granules (2-6 µm in diameter) are polygonal and smaller than maize starch granules (5–30 µm in diameter) (Figure 2.1) (Bultosa, 2003). Granules of tef have compound structures comprised of individual granules (Figure 2.2) similar to that of rice and amaranthus (Bultosa *et al.*, 2004). Scanning electron micrographs showed no pores on the surface of tef starch granules (Bultosa, 2003). Maize starch granules were found to contain pores (Fortuna *et al.*, 2000). These pores were observed to be openings to channels penetrating the external granule surface into the interior (reviewed by Huber & BeMiller, 2000). The channels on maize starch granules were found to be lined with proteins and phospholipids (Han & BeMiller, 2008).

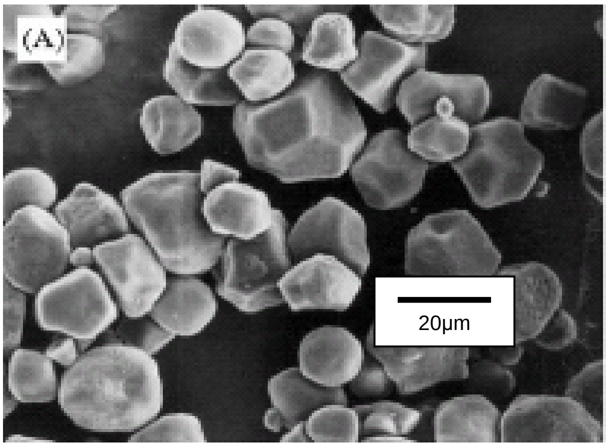


Figure 2.1 Native maize starch granules (Singh *et al.*, 2007)

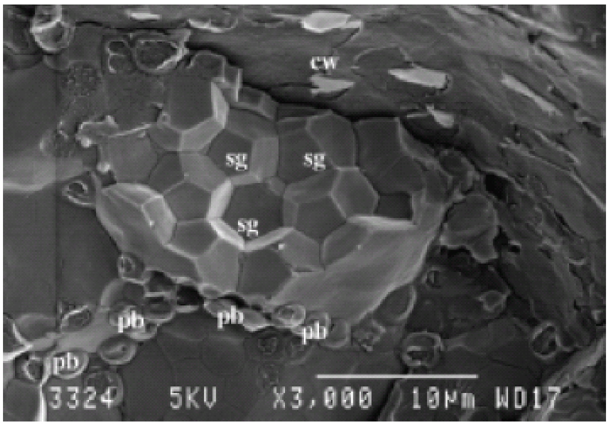


Figure 2.2 Compound tef starch granule. sg = individual starch granule, pb = protein bodies, cw = cell wall (Bultosa, 2003)

2.1.2 Starch polymers

Both starch polymers, amylose and amylopectin, are composed of α-D-glucopyranosyl units, but they differ in size, shape and functionality. The amylose content of tef starch has been reported to be around 28%, typical to that of other cereal starches, such as maize and sorghum (Bultosa & Taylor, 2004). Amylose molecular weight was found to be similar for tef and maize starches (Bultosa *et al*., 2008). The average molecular weight for tef amylopectin (13.9×10^7 g/mol) determined using multi angle laser light scattering-differential refractive index detection (SEC/MALL-DRI) is reported to be considerably smaller than that of maize amylopectin (19.6×10^7 g/mol) (Bultosa *et al.,* 2008).

2.1.2.1 Amylose

The amylose molecule (Figure 2.3a) is mostly linear and consists of α (1-4) linked D-glucopyranosyl units (Zobel *et al*., 1988a). The amylose molecule may hold between 9–20 branches, with chain lengths of between 4–100 anhydroglucose units (reviewed by Oates, 1997). Amylose molecules have been reported to have between 100-10000 linked anhydroglucose units (reviewed by Hermansson & Svegmark, 1996) and a molecular weight ranging between 1.6×10^5-7.1×10^5 g/mol (Kent & Evers, 1994). Amylose is in the form of a double helix that consists of two single helical amylose chains folded parallel around each (Bajner, 2002; Immel & Lictenhaler, 2000). There are two crystalline polymorphic arrangements of double helical amylose in the starch granule, namely A and B amylose (Rappenecker & Zugenmaier, 1981; Immel & Lictenhaler, 2000 and Tester *et al.*, 2004). The helical structure of the two polymorphs is basically identical, but there packing within the crystalline structure differ (Tester *et al.*, 2004). The A type structure is compact with a low water content while the B type has a more open structure containing a hydrated helical core (Tester *et al.*, 2004). Single helical amylose (Figure 2.3 b) has six glucose units per turn and mostly exist in the form of inclusion complexes with di-methylsulphoxide, *n*-butanol and *n*-pentanol or in a dry (V_A-amylose) or hydrated (V_H-amylose) form (Rappenecker & Zugenmaier, 1981).

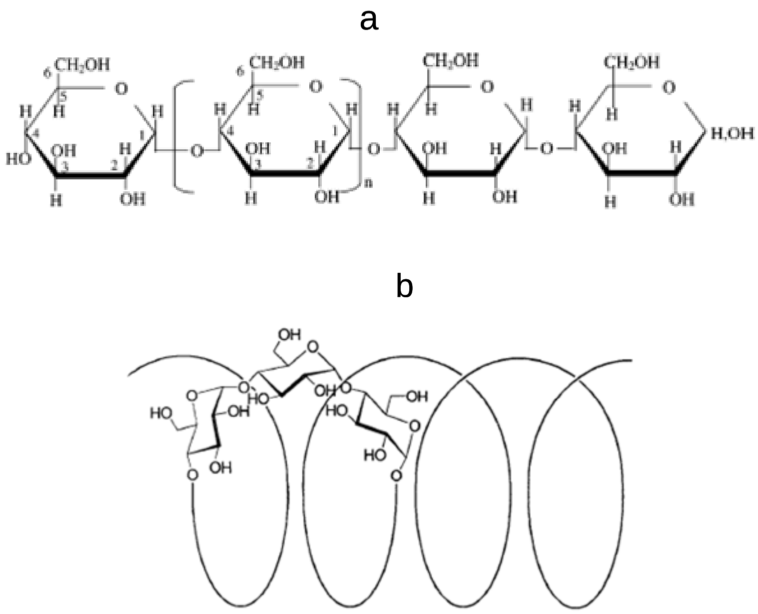


Figure 2.3 (a) Part of straight chain of amylose showing the α (1-4) linked D-glucopyranosyl units (reviewed by Tester *et al*., 2004); **(b)** Single amylose helix (Immel & Lictenhaler, 2000)

2.1.2.2 Amylopectin

The amylopectin molecule has been reported to be structurally as well as functionally, the more important starch fraction (reviewed by Oates, 1997). Amylopectin (Figure 2.4a) is a heavily branched molecule made up of D-glucopyranosyl units joined by α (1-4) glycosidic bonds to form the linear chain and α (1-6) linked branch points (Gunaratne & Corke, 2004). Amylopectin is larger than amylose with an average molecular weight of 10^8 (Jackson, 2003). The amylopectin molecule is considered to consist of three types of chains known as the A, B and C chain. The A chains are unbranched side chains that are attached to the rest of the molecule by their reducing ends (Kent & Evers, 1994). B chains are branched and connected to other chains (reviewed by Oates, 1997) and the chains to which the A chains are attached (Parker & Ring, 2001). The C chain carries the only reducing group of the molecule (Parker & Ring, 2001). Figure 2.4(b) shows the A and B chains of the amylopectin molecule.

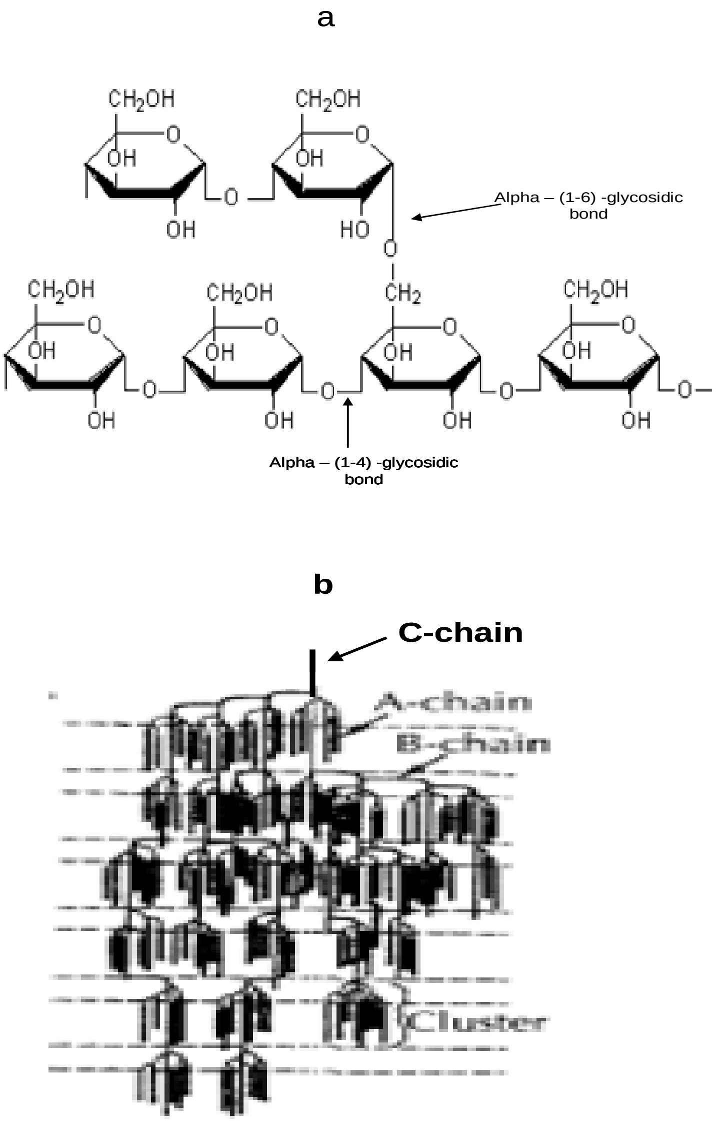


Figure 2.4 (a) Part of the branched chain of the amylopectin molecule; **(b)** amylopectin molecule showing the A & B chains (Oates, 1997)

2.1.3 Starch granule organization

A great deal of research has been conducted on the starch granule structure and one of the probable models developed is the “cluster” or “blocklet” model (Ohtani *et al.*, 2000). Figure 2.5 (a-d) illustrates the theory of the “Blocklet” model. Atomic force microscopy (AFM) images of the starch granule showed the presence of nodules termed as blocklets (Figure 2.5c) (Gallant *et al*., 1997). It is proposed that the assembly of two or three layers of blocklets form the alternating semi-crystalline and amorphous growth rings (Figure 2.5b) (Gallant *et al.*, 1997). Blocklets contain several amorphous and crystalline lamellae (Figure 2.5d). The lamellae consist of double helical amylopectin side chain clusters. Figure 2.6 shows the amylopectin side chain clusters within lamellae. The intertwining of the amylopectin chains form double helices (reviewed by Oates, 1997). The crystalline clusters alternate with more amorphous lamellae that are made up of the amylopectin branching regions (Gallant *et al.*, 1997) and contains free amylose and amylose complexed with lipids (Gunaratne & Corke, 2004).

2.1.4 Starch granule crystallinity

The starch granule crystallinity is between 15% and 45% (Zobel, 1988). Amylopectin is mainly responsible for the crystalline order of the starch granule (reviewed by Hermansson & Svegmark, 1996; Gallant *et al.*, 1997). In order to detect the presence and type of crystallinity in native starch granules, X-ray diffraction analysis has been useful. Native starch is categorized into three types, namely A, B and C according to its X-ray diffraction patterns (Zobel, 1988; Kent & Evers, 1994). In starch crystalline structure, the A-type structure is more compact and consists of six bundles of double helical starch molecules packed radially and a seventh bundle of double helices filling the central space The B-type is a more open and hydrated structure (reviewed by Parker & Ring, 2001) and consists of six bundles of double helices packed in a hexagonal manner, with the granule centre part filled with water molecules.

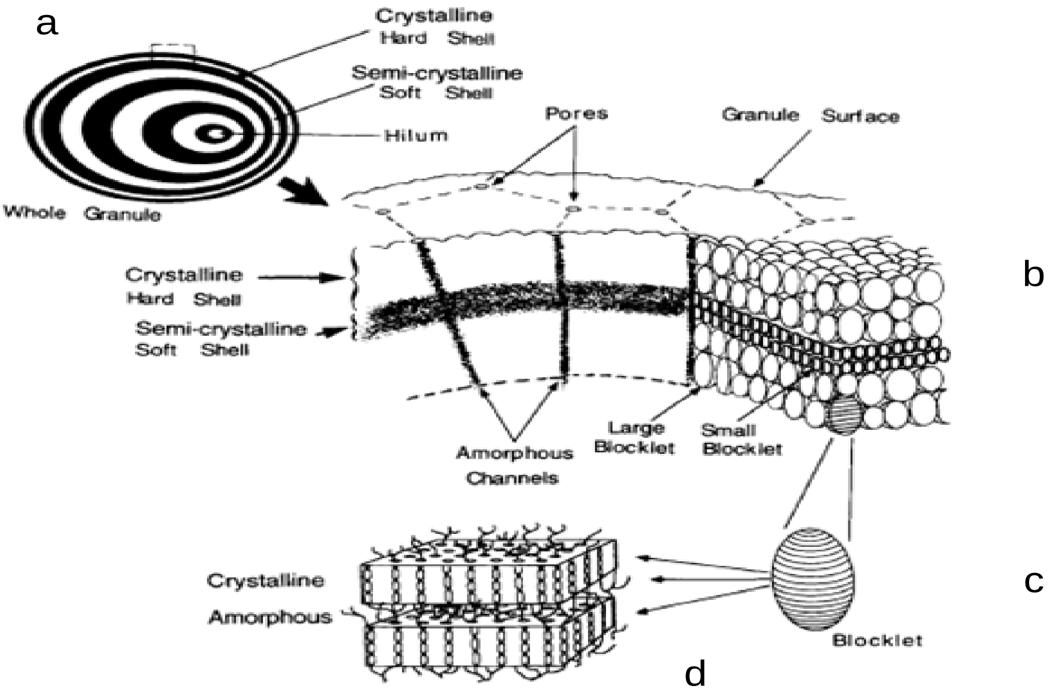


Figure 2.5 Overview of the starch granule structure; **(a)** whole starch granule with alternating crystalline and amorphous regions; **(b)** assembly of blocklets to form layers; **(c)** one blocklet is shown; **(d)** to contain several amorphous and crystalline lamellae (Gallant *et al.*, 1997)

The C-type is found in tubers and grain legumes and may be a mixture of both the A and B form. A fourth V-type crystalline form is typically shown when the amylose fraction form complexes with molecules such as alcohols, fatty acids and iodine (Zobel, 1988b). Most cereal starches have the A-type crystallinity (Zobel, 1988b) indicating that they contain densely packed crystallites (Gallant *et al.*, 1997). The crystalline level is said to be influenced by the length of the amylopectin A chain, the amount of double helices organized in a crystalline array, crystallite size and amylose content (Bultosa & Taylor, 2003). Bultosa *et al.* (2003) reported that the level of crystallinity of tef starch was lower than that of maize starch and therefore similar to sorghum and rice starch. The use of an X-

Ray diffractometer to analyze starch crystallinity produces diffractograms as shown in Figure 2.7.

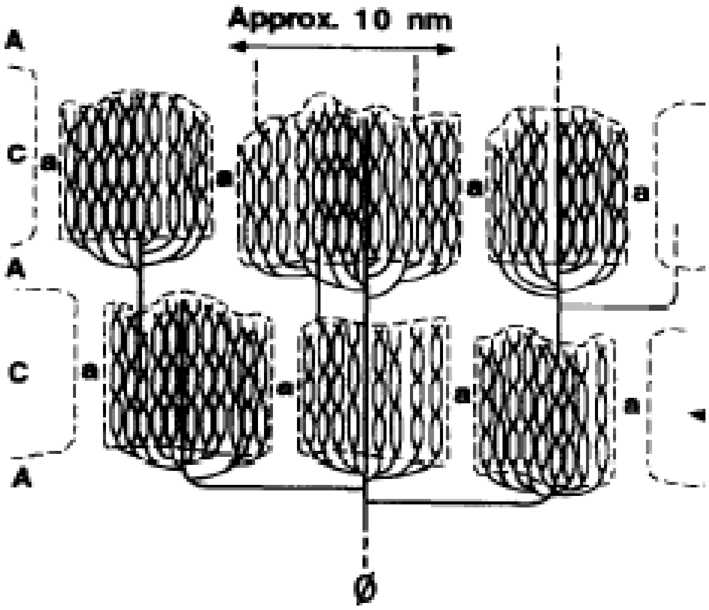


Figure 2.6 Amylopectin side chain clusters within lamellae. A, amorphous lamellae and C, crystalline lamellae (Gallant *et al.*, 1997)

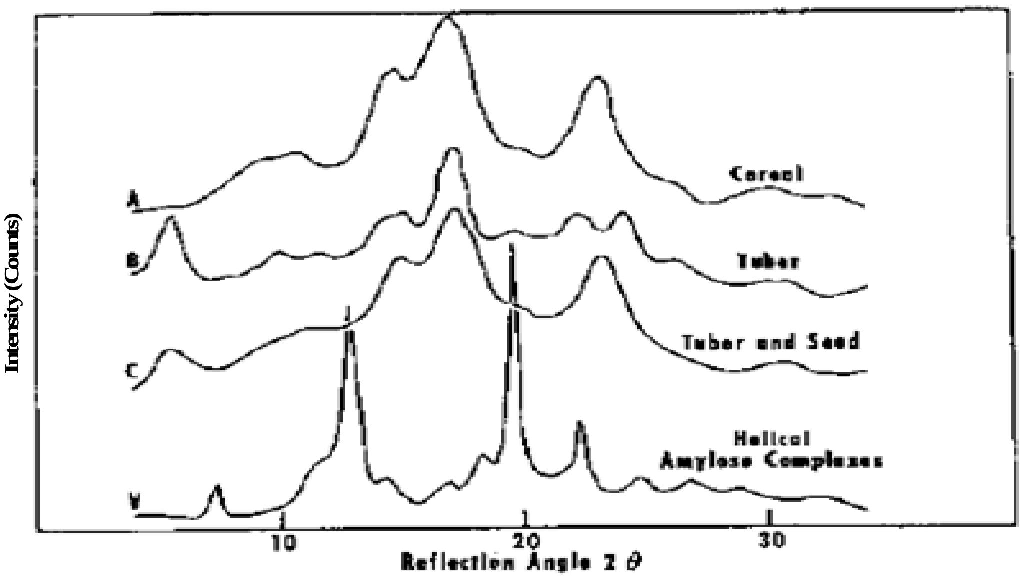


Figure 2.7 X-Ray diffractograms for different starches (Zobel, 1988b)

2.2 Starch granule gelatinization, pasting and retrogradation

Native starch granules are insoluble in cold water (Thomas & Atwell, 1999). Gelatinization, pasting and retrogradation are three phenomena that occur during the heating of starch (above 60°C) in the presence of water (Thomas & Atwell, 1999; Lui, 2005). Changes in the starch granules involve swelling of the granules and exudation of the molecular components, especially amylose. This irreversible swelling and disruption of starch granules results in the development of viscosity. The thermal behaviour of starch granules can be evaluated using various instruments, one being a Rapid Visco Analyser (RVA) (Thomas & Atwell, 1999). The RVA is an instrument that measures change in viscosity over time while stirring and heat is applied. The viscosity measurement of starch slurry using the RVA will produce a pasting curve. Figure 2.8 shows the pasting curves of different tef varieties and a maize variety.

The phases of starch pasting can be identified from looking at the pasting curves of tef and maize starch (Figure 2.8). Heating of starch in the presence of water to a characteristic temperature range, results in irreversible granular swelling and viscosity development, due to the uptake of water into the starch granules (reviewed by Parker & Ring, 2001). These changes are accompanied by crystallite melting (Kent & Evers*,* 1994) and starch solubilization (Thomas & Atwell*,* 1999; Gunaratne *et al.*, 2004). This transformation, during which the starch granule loses molecular order and birefringence, is termed gelatinization (reviewed by Bultosa, 2003; Kent & Evers, 1994). Every starch granule does not gelatinize at exactly the same temperature (Thomas & Atwell, 1999). According to Bultosa *et al.* (2002) the gelatinization temperature range for tef starch, 68°C (onset), 74°C (peak) and 80°C (conclusion), measured using a hot stage polarized light microscope system is similar to that of maize starch, 65°C (onset), 73°C (peak) and 80°C (conclusion).

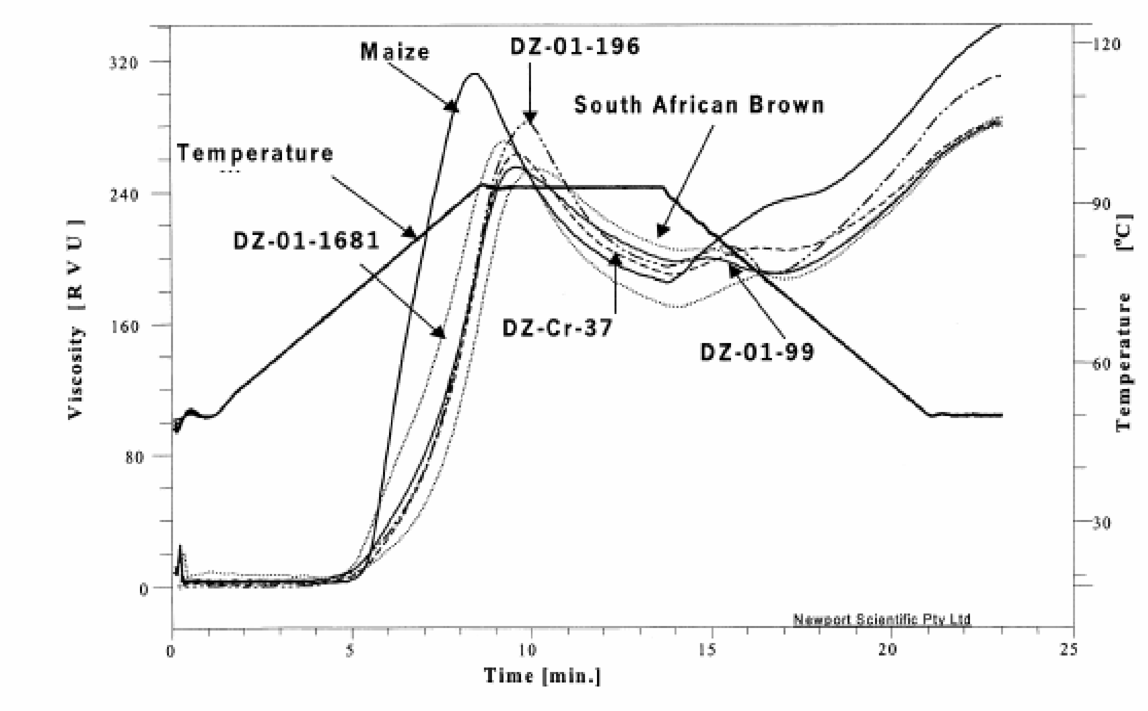


Figure 2.8 Pasting curves of different tef varieties and a maize variety (Bultosa, 2003)

As heating continues, starch granules continue to swell until a maximum viscosity is reached (peak viscosity). This phase that follows gelatinization is known as pasting (Thomas & Atwell, 1999). During pasting there is a disruption of the starch granules and the starch molecules that leach into the solution are solubilised (Gunaratne & Corke, 2004). Gelatinized starch granules that are not solubilised form part of the discontinuous starch paste and are referred to as ‘ghost’ granules (Whistler & BeMiller, 1997). Continued stirring of the starch paste causes a decrease in viscosity. This is primarily because the solubilised polymers align themselves in the direction of stirring (Whistler & BeMiller, 1997) as well as further granular breakdown (Jackson, 2003).

A second viscosity increase after breakdown, known as setback, is observed upon cooling of the starch paste (reviewed by Kent & Evers, 1994). This is the result of the reassociation of starch molecules in order to re-establish ordered structure and is referred to as retrogradation (Thomas & Atwell, 1999). Upon cooling, entangled amylose molecules trap water in their three-dimensional gel network, leading to the formation of crystallites that cause the paste to increase in viscosity (Kent & Evers, 1994). Linear, unbranched amylose molecules are more likely to reassociate and form hydrogen bonds (Thomas & Atwell, 1999) due to less steric hindrance during reassociation. Therefore initial retrogradation is largely due to the crystallization of amylose (Zobel *et al.*, 1988a). Amylopectin retrogrades slower possibly because of its larger and branched structure (reviewed by Parker & Ring, 2001).

Retrogradation, among others, is regarded as a limitation of native starch for certain food application, for example the staling of bread (Karim *et al*., 2000). Starch is therefore modified in order to overcome this drawback.

2.3 Modification of Starch

Starch modification has been proven successful in improving its functionality and greatly increasing its usefulness (Aggarwal & Dollimore, 1998). Starch modification involves altering its physical and chemical characteristics thereby improving functions such as thickening, binding, stability, mouthfeel, gelling, dispersion or cloud formation (Singh *et al.*, 2007). Starch can be modified physically and chemically.

Physical modification involves the use of a physical processing technique to overcome the limitations of native starch (Thomas & Atwell, 1999). The major reason for the physical modification of starch is to bring about a change in the granule structure of the starch or to convert the starch into cold-water soluble starch or small crystallite starch. There are various methods used for the physical modification of starch, depending on the desired altered starch properties. Table 2.1 summarizes some methods used for physical starch modification. Chemical modification is achieved by the use of chemicals and is regarded a mainstream of modified starch and many types of chemical modifications have been developed and applied in the food, paper and textile industries (Miyazaki *et al.,* 2006). This type of modification (depending on the chemicals used) improves starch properties such as freeze-thaw stability, paste clarity and reduced retrogradation. Table 2.1 summarizes chemical modifications most often applied. Starches used for modification are maize, waxy maize, tapioca, potato, sorghum, wheat, rice and sago.

Health and sensory concerns from consumers and legislative organizations, regarding the use of chemicals to modify starch, have created the possibility of using natural compounds, for example fatty acids. Fatty acids, among other chemical constituents (for example flavour compounds and emulsifiers) are able to interact with the hydrophobic interior of the amylose helix to form inclusion complexes (Jackson, 2003; Raphaelides & Karkalas, 1988).

Fatty acids are a group of lipids that are characterized by having polar head groups (carboxyl group) and non-polar tails (hydrocarbon chain). Fatty acids are amphipathic, which means that their structure includes both hydrophilic and hydrophobic parts. Maize starch native lipid components are mainly free fatty acids (FFA's) and monoglycerides (MG) (i.e. lysophospholipids) (Morrison, 1995). Stearic acid is a naturally occurring saturated fatty acid with 18 carbon atoms.

2.4 Interactions of fatty acids to modify starch properties

The interaction of fatty acids with starch has been found to alter starch properties by reducing granular swelling, peak viscosity and retrogradation (Godet *et al*.,1995; Jackson, 2003 and Zhou *et al*., 2007), reduced susceptibility to enzyme (α-amylase) digestion (Kaur & Singh, 2000) and improving freeze thaw stability (Godet *et al*., 1995). The following section (Section 2.4.1) reviews studies showing the effects of modifying starch with fatty acids on its functional properties and structure.

Table 2.1 Summary of some physical and chemical modifications and their effect on starch properties

Modification	Types	Chemistry	Effect on functional and/or pasting properties
Physical	Pregelatinisation, Heat and High Pressure (above 400 MPa)	--------------	Viscous starch pastes,improved solubility [a,b]
Chemical	Etherification with Propylene Oxide and Esterification with Acetic Anhydride / Vinyl Acetate/ Octenyl succinic anhydride	Chemical agent substitutes the hydrogen on the anhydroglucose-unit preventing alignment of the polysaccharide chains and interfering with aggregation	Increased paste clarity, decreased pasting temperatures and reduced retrogradation and gelling upon ageing [c,d,e] increased sol clarity [d] Increased cold-water solubility reduced gelatinization temperature, increased freeze-thaw [g,h]
	Phosphorylation with Orthophosphate / Sodium tripolyphosphate	Reagent group binds to the hydroxyl group, forming a monoester. Bulky group interferes with alignment and imparts anionic charge leading to starch molecules repelling each other	Stability at low temperatures, increased viscosity, improved freeze-thaw stability and improved paste clarity [i,j]
	Crosslinking with Phosphorus Oxychloride / Sodium trimetaphosphate	Chemical agent introduces bridges between the starch molecules that reinforces and strengthens the granule	Increased stabilization at extreme temperatures, acidity and shear. Reduced paste clarity and reduced cold storage stability is a shortcoming of crosslinked starch [a,k]

[a] Miyazaki *et al.* (2006) [b] Waliszevski (2002) [c] Gunaratne *et al.* (2007) [d] Raina *et al*.(2006) [e] Wang & Wang (2000) [f] Adebowale *et al.* (2005) [g] Betancur-Ancona *et al.* (2002) [h] Bhandari & Singhal, (2001) [i] Wurzburg (2006) [j] Whistler *et al* . (1984) [k] Singh *et al.* (2007)

2.4.1 Effects of fatty acids on starch pasting behaviour

The addition of fatty acids to starch impacts on starch pasting behaviour. The addition of stearic acid (C18:0) and linoleic acid (C18:2) to rice starch was found to alter its pasting properties, with saturated stearic acid having a more pronounced effect (Zhou *et al.*, 2007). Changes included decreased peak and breakdown viscosity and increased time to peak viscosity and final viscosity, measured using the Rapid Visco Analyser (set to a pasting profile of 15 min at a constant stirring rate of 160 rpm). Raphaelides & Geogiardis (2006) found that myristic, palmitic and stearic acid in maize starch dispersions, heated in a rheometer to up to 95°C (2.1°C/min with constant stirring 40 to 60 rpm) retarded granular swelling (measured with a laser particle size analyzer) thereby slowing down gelatinization, reducing peak viscosity and increasing the time to peak viscosity. Similar trends were found for maize starch by Singh *et al.* (2002). Bajner (2002) also found the addition of stearic acid to maize starch reduced peak viscosity and increased time to peak viscosity.

In an investigation by D'Silva (2009), stearic acid was added to tef and maize starches in different concentrations (0.25%, 0.5%, 1%, 1.5%, 2%, 3% and 4%) followed by pasting in the RVA. The trend for maize starch results was similar to results observed by Raphaelides & Georgiadis (2006) (for maize starch) and Tang & Copeland (2007) (for wheat starch), showing reduced peak viscosity and increased time to peak viscosity with addition of stearic acid (D'Silva, 2009). Tef starch results proved unusual in that no first peak viscosity was reached within the first 15 min, when stearic acid concentration was increased. Tef starch pastes with added stearic acid increased in viscosity during the holding period until temperatures were reduced. Increased stearic acid concentration increased final viscosity for tef starch. It was suggested by Richardson *et al*. (2003) that monoglycerides form a layer around granules, thereby increasing their hydrophobicity. This would result in the granules taking up less water and thereby reduce granule swelling, giving a reduced peak viscosity. The findings of

D'Silva (2009) further supported what Richardson *et al*. (2003) suggested. D'Silva (2009) observed stearic acid on the surface of tef starch granules (using confocal laser scanning microscopy) and not inside the granules as was the case with maize starch. She attributed the unexpected pasting behaviour of tef starch to the observation that the stearic acid could not diffuse into the granules as it did not have any pores on the surface (Bultosa *et al*., 2002).

Richardson *et al*. (2003) investigated the effect of a mixture of poly glycerol-ester and a monoglyceride (PGE/MG) on wheat starch suspensions. They added PGE/MG in concentrations 0.5% and 4% and the suspensions were held at various temperatures. The control samples showed amylose leaching at 70°C and fragmentation of the granules at 90°C while the addition of PGE/MG almost completely restricted starch swelling above 50°C and amylose leaching was delayed until about 90°C. Some granules were still intact at 90°C, but above 90°C complete granule rupture occurred.

D'Silva (2009) subjected stearic acid modified tef and maize starches to an extended pasting profile and observed the development of a second pasting peak during the holding period (2 hours at 91°C). The development of a second pasting peak was also observed by Nelles *et al.* (2000) (Figure 2.9) and Bajner (2002) when native maize starch was subjected to an extended pasting profile (with a total time of approximately 110 min at 91°C) in the RVA. This second viscosity peak has been attributed to the formation of starch-lipid complexes (Nelles *et al*., 2000). This is because the second viscosity peak was not observed when the lipid fraction of the starch was removed but re-appeared when the fat removed was added back (Nelles *et al*., 2000). The second peak was found to increase with the addition of fatty acids (Bajner, 2002; D'Silva, 2009).

To further explore the theory of the role of amylose-lipid complexes in the formation of the second peak, Nelles *et al*., (2003) used scanning electron microscopy (SEM) to investigate samples of starch pastes containing lipids, taken at various intervals throughout extended pasting. They observed that after the first peak, starch granules were swollen and still intact with some leaching (Figure 2.10a & b). After the second peak, the granule integrity was lost and there was only a homogenous mass present (Figure 2.10d). HPLC analysis showed there to be a decrease in the amylose content of the starch pastes during the second peak maximum and the breakdown of the second peak, compared to higher solubilised amylose during the other periods of pasting (Nelles *et al*., 2003). They suggested that this decrease in solubilised carbohydrates as well as the intact granules up to the second peak development was due to amylose interacting with lipids to form amylose-lipid complexes (Nelles *et al*., 2003). Although this evidence further confirms the collective roles of amylose and lipids in the development of the second peak and the possible effect of lipids on starch microstructure, it does not prove that the second peak is a result of the formation of amylose-lipid inclusion complexes, since no crystalline complexes were observed by these authors.

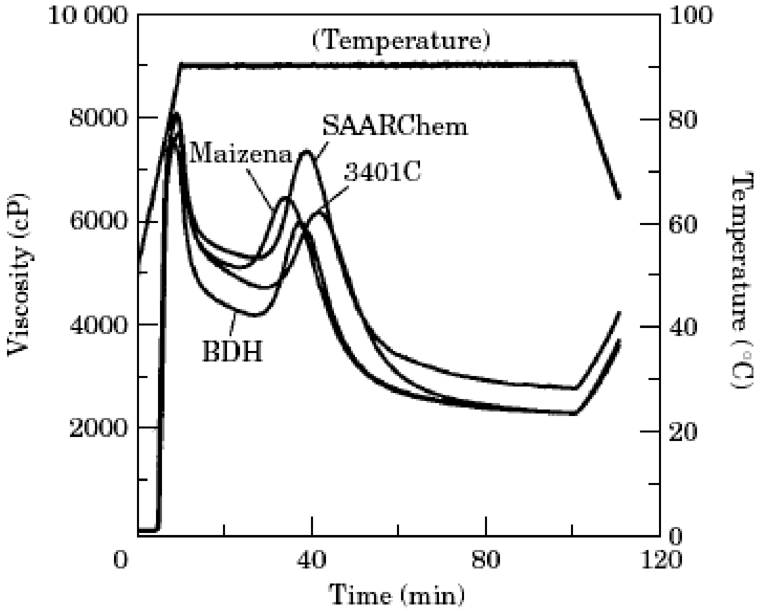


Figure 2.9 Pasting curves of different native maize starches (BDH, maizena, SAARChem and 3401C) showing the second increase in viscosity (Nelles *et al.*, 2000)

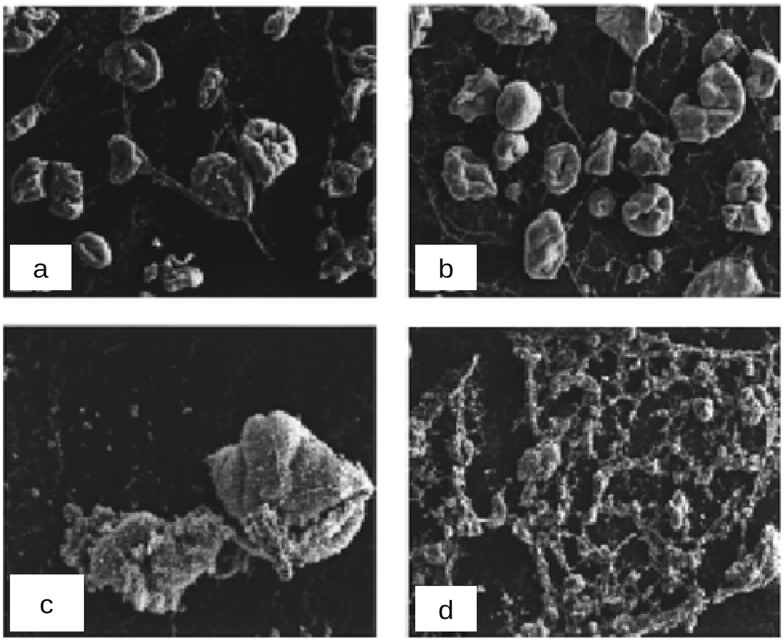


Figure 2.10 Scanning electron micrographs of maize starch pasted for a - 10 min × 500 ; b – 22.5 min × 500; c – 34 min × 2500 & d - 95 min × 500 (Nelles *et al*., 2003)

Amylose-lipid complexes, prepared by jet cooking, were isolated and investigated using microscopy analysis, X-ray diffraction and differential scanning calorimetry (DSC), in the following studies. Although these investigations were conducted on maize and potato starches and using steam jet cooking for the preparation of the amylose-lipid complexes, similar effects could possibly be found for tef and maize starches modified with stearic acid during extended pasting to produce a second viscosity peak.

Fanta *et al*. (2002) and Peterson *et al.* (2005) found that upon cooling of steam-jet cooked starch solutions helical inclusion complexes of amylose with native starch granular lipids, particularly fatty acids (Byars *et al.*, 2003), crystallized to form spherocrystals. Spherocrystals were not formed from waxy cornstarch, defatted normal maize starch or potato starch (Fanta *et al.,* 2002). They therefore concluded these spherocrystals to be amylose-lipid complexes. The method of formation of these spherocrystals was termed to be “high temperature retrogradation", as a high processing temperature and a high storage temperature were required for crystal formation (reviewed by Byars *et al*., 2003). After lipid extraction from the spherocrystal samples, the absorbance of the amylose iodine complex was determined calorimetrically and showed the spherocrystals aggregates to be composed of mainly amylose (Fanta *et al.,* 2002).

Spherocrystals formed were reported to consist of two types, small and large, that differed in size, morphology, crystal structure and properties (Fanta *et al.*, 2002). Both species of spherocrystals were birefringent (Figure 2.11 C and D), even after being heated to between 97-99°C, in the presence of water (Fanta *et al.*, 2002). Figure 2.11 shows micrographs of small and large spherocrystals formed from maize starch (Fanta *et al*., 2002). The small crystals (Figure 2.11 B, D & F) are disc or torus shaped less than 10 µm in diameter and more uniform. The larger crystals (Figure 2.11 A, C & E) are spherical in shape and between 10-30 µm in diameter (Byars *et al.*, 2003). The size, shape and amount formed vary with experimental conditions and minor variations in cooking and cooling (Fanta *et al.*, 2002).

X-ray diffraction patterns of the spherocrystals showed d-spacings of 11.8 Å, 6.86 Å and 4.48 Å. These d-spacings are typical of V-type crystallites (Fanta *et al.,* 2002; Fanta *et al.,* 2005) which is also characteristic of amylose-lipid inclusion complexes. The X-ray diffractogram for large crystals indicates that the amylose helix is larger in diameter because of complex formation with a bulkier, unsaturated lipid (Fanta *et al.,* 2002). The X-ray diffractogram suggested that the structure of the spherocrystals may be governed to a large extent by the chemical structures of the lipids that make up the complexes (Fanta *et al.,* 2002). After pasting wheat starch with stearic acid Tang & Copeland (2007) found the X-ray diffractogram matched the V-type pattern. They also observed additional peaks at 2θ = 21.5° and 23.9°, which were identified as the crystalline pattern of stearic acid.

In order to determine if a spherocrystal with a single size and morphology could be produced, native fatty acid were replaced with pure stearic acid (18:0). The result were 80% large and 20% small crystals (Fanta *et al.,* 2006) indicating higher molecular weight fatty acids are found more abundantly in large crystals (Peterson *et al.*, 2005). Figure 2.12 shows micrographs of the large and small crystals formed with the addition of stearic acid to maize starch.

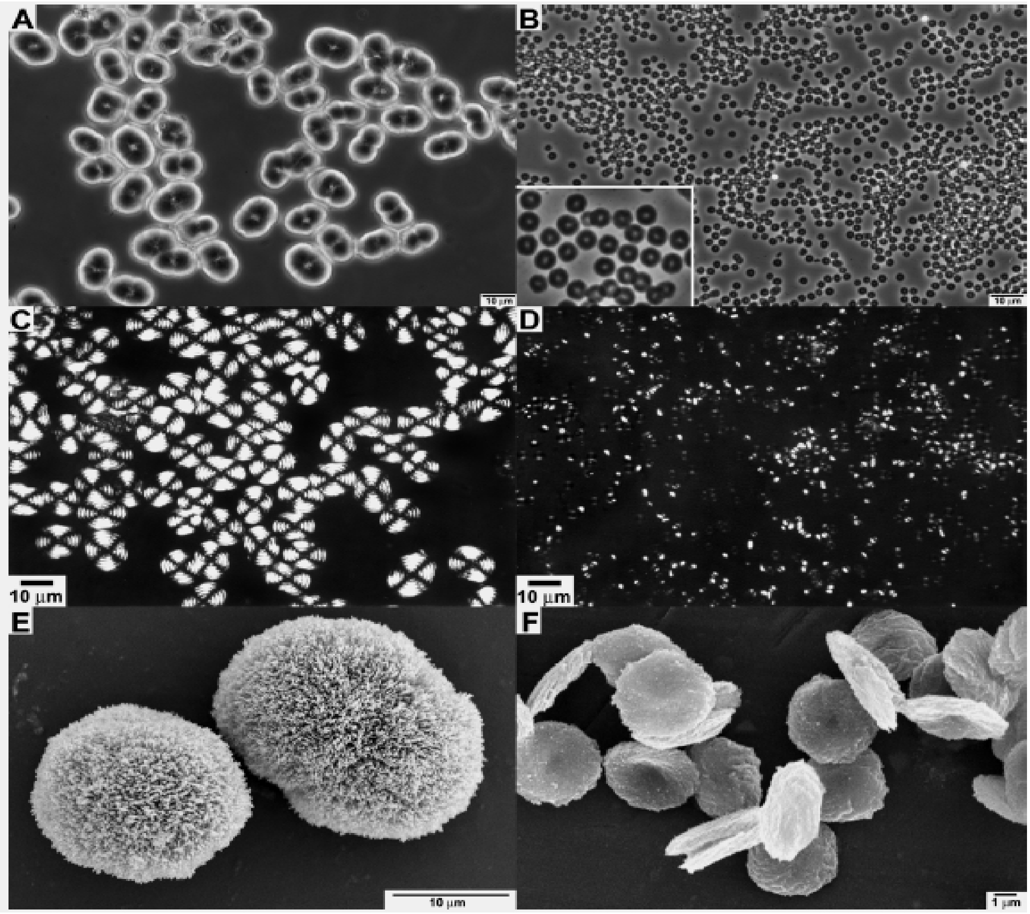


Figure 2.11 Micrographs of spherocrystals formed from normal cornstarch; phase contrast micrograph of large particle crystals **(A)** and **(B)** small particle crystals; **(C)** & **(D)** same as above but viewed with polarized light; SEM of large particles crystals **(E)** and small particle crystals **(F)** (Fanta *et al*., 2002)

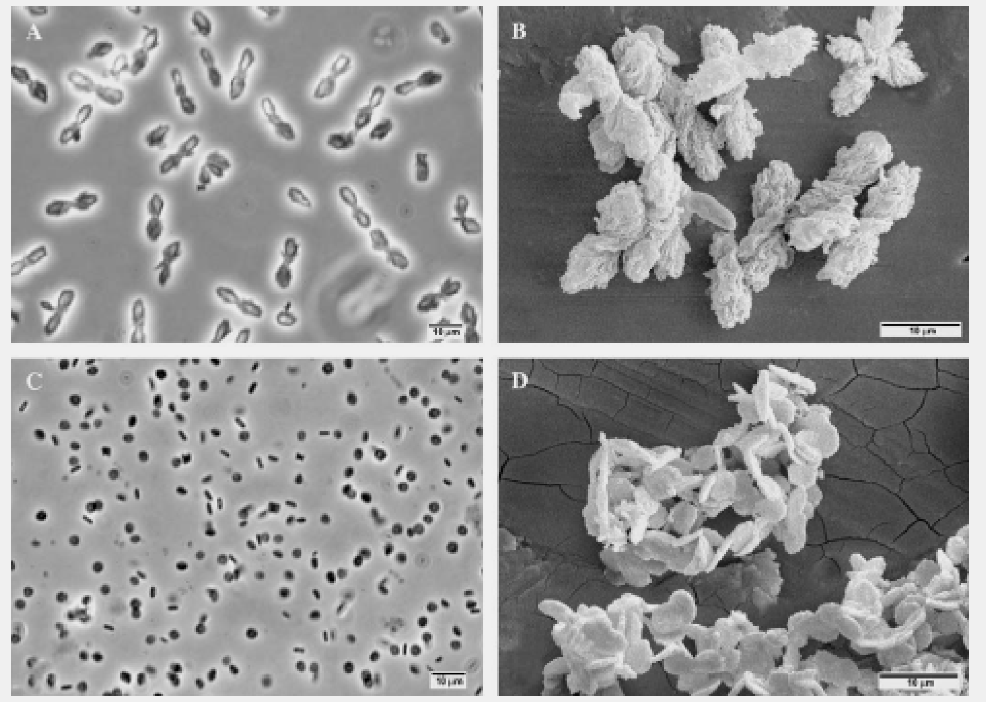


Figure 2.12 Micrographs of spherocrystals formed with stearic acid and defatted cornstarch; (A & C) Phase contrast images, (B & D) SEM images, (A & B) large crystals, (C & D) small crystals (Fanta *et al.*, 2006)

Formation of the spherocrystals is affected by various factors such as cooling rate, starch concentration and starch type (Fanta *et al.*, 2005). Spherocrystal formation and isolation favoured slow cooling rate as rapid cooling inhibited formation and a strong gel formed (Byars *et al.*, 2003). High starch concentrations lead to phase separation of amylose and amylopectin, making amylose more available for complexing and resulting in increased amounts of larger crystals (Fanta *et al.*, 2005). Crystalline particles were formed from dispersions of normal maize starch, high amylomaize starch, corn starch and wheat starch but not from dispersions of waxy maize starch, defatted maize starch and potato starch (Fanta *et al.*, 2002). As spherocrystals are reported to be crystallized helical inclusion complexes formed form amylose and lipids, the absence of amylose in waxy maize starch and the low lipid concentrations in defatted maize (without addition of exogenous lipids) and potato starch (0.05%)

substantiates there inability to produce spherocrystals (reviewed by Fanta *et al*., 2002).

2.5 Chemistry of amylose-lipid complex

The effect of fatty acids on starch pasting behaviour, especially the development of the second peak during extended pasting and spherocrystal formation, is attributed to the formation of amylose-lipid inclusion complexes. Inclusion complexes are formed through the interaction of the hydrophobic interior of the amylose helices with guest molecules, such as flavour compounds, iodine, alcohols, fatty acids, mono- and diglycerides (Jackson, 2003; Godet *et al.*, 1995). Amylose-lipid complex formation generally occurs in starch containing natural lipids or with the addition of lipids to defatted starch (Godet *et al.*, 1993). The complex is formed when the aliphatic fatty acid tail occupies the central hydrophobic core of the amylose helix, consisting of six, seven or eight glucose units per turn (Karkalas & Raphaelides, 1986). Steric hindrance and electrostatic repulsion prevents the polar fatty acid head form being incorporated into the amylose helix (Godet *et al.*, 1993). The hydrophobic tail of the fatty acid is stabilized by the hydrophobic interaction and Van der Waal forces between the hydrogen atom on the fifth carbon of the glucopyranose unit of amylose and the hydrogen atoms linked to each carbon of the fatty acid chain (Godet *et al*., 1993; Siswoyo & Morita, 2003). The fatty acid chain is immobilized within the amylose helix (Morrison, 1995). Amylose complexation requires the monomer form of the lipid (Eliasson & Krog, 1985). Saturated monoglycerides with fatty acid chain lengths from C_{12} to C_{18} are very effective amylose complexing compounds (Eliasson & Krog, 1985). The cis-double bonds (C=C) of unsaturated fatty acid chains obstruct their access to the helical amylose core (Kaur & Singh, 2000). They are however able to complex with amylose through free rotation of adjacent single bonds (C–C) (Karkalas *et al*., 1995).

Figure 2.13 illustrates how the stearic acid tail (carbon chain) is included into the amylose helix, consisting of six glucose units per turn, where it takes up 3 complete turns of the helix. The polar carboxylic head of stearic acid is unable to enter the helix (Carlson *et al.*, 1979)

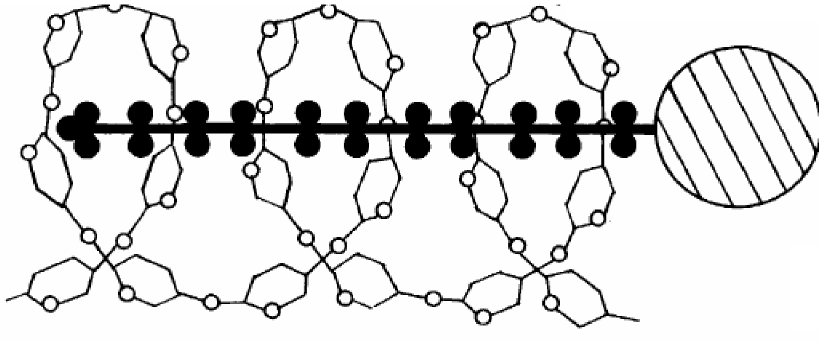


Figure 2.13 Schematic representation of a monostearin-amylose complex, the hydrogen atoms of the fatty acid are indicated (Carlson *et al.*, 1979)

X-ray diffraction studies show the amylose-lipid complex to have a lamella–like organization (Biliaderis & Galloway, 1988). The amylose lipid complex exhibits the V form crystallinity (Zobel *et al.*, 1988b) and is therefore given the name: V-amylose (Godet, 1993). The V-amylose structures are formed when A and B-type double helical structures collapse and yield a single left-handed helix (Rappenecker & Zugenmaier, 1981). The crystalline structure of the V-amylose complex can be isolated as hexagonal, rectangular or square platelets (reviewed by Kim & Lim, 2009). V-amylose exists in two forms namely, dry (V_A) and hydrated (V_H) (Rappenecker & Zugenmaier, 1981). There are two water molecules inside the V_H–amylose that are bound to each other, but not to the helix, because of its hydrophobic interior (Rappenecker & Zugenmaier, 1981). For the hydrated V form (V_H) peaks at 2θ values appeared at 12°, 6.8° & 4.4°. The dehydrated V form (V_A) shows reduced spacings at 11.3°, 6.5° & 4.3° (Zobel *et al.*, 1988). X-Ray analysis on freeze-dried wheat starch mixed with stearic acid (0.05 mmol) produced 2θ peaks (7.4°, 12.7° & 19.8°) which matched the V pattern observed for amylose-lipid complexes (Tang & Copeland, 2007). The peaks were reduced when the amount of stearic acid was increased to 0.14 mmol. Above certain concentrations, lipids with low water solubility (like stearic and palmitic acid) associate with themselves rather than form complexes with amylose helices (Tang & Copeland, 2007).

Two types of amylose-lipid complexes have been identified, namely type I and type II complexes.

Differential scanning calorimetry methods have been used for the identification of type I and type II amylose-lipid complexes (Kugimiya *et al.,* 1980; Karkalas *et al*., 1995 and Tufvesson *et al*., 2003). Figure 2.14 depicts the possible mechanism for their formation.

At temperatures below gelatinization (low T_c) and in the presence of a good complexing agent, nucleation rate is high and type I complex formation is favoured. The structure for these complexes is described as being a random distribution of helical segments (Kaur & Galloway, 1989). These complexes are insoluble and amorphous and dissociate upon heating in water at 94–104 °C (Raphaelides & Karkalas, 1988; reviewed by Morrison, 1995). Karkalas *et al*. (1995) reported the type I amylose-stearic acid complexes formed at below 60°C and dissociated at between 96-104°C. Type I complexes lack a well defined V-pattern (Biliaderis & Galloway, 1989). Type I complexes can be annealed to a semi-crystalline form (Type II) (reviewed by Morrison, 1995).

Type II complexes are suggested to be superstructures of several type I complexes crystallized together by hydrogen bonds and van de Waals forces (Biliaderis & Galloway, 1989). Type II complexes form after gelatinization (reviewed by Morrison, 1995). These complexes dissociate at 100–125°C (Zobel, 1988b).

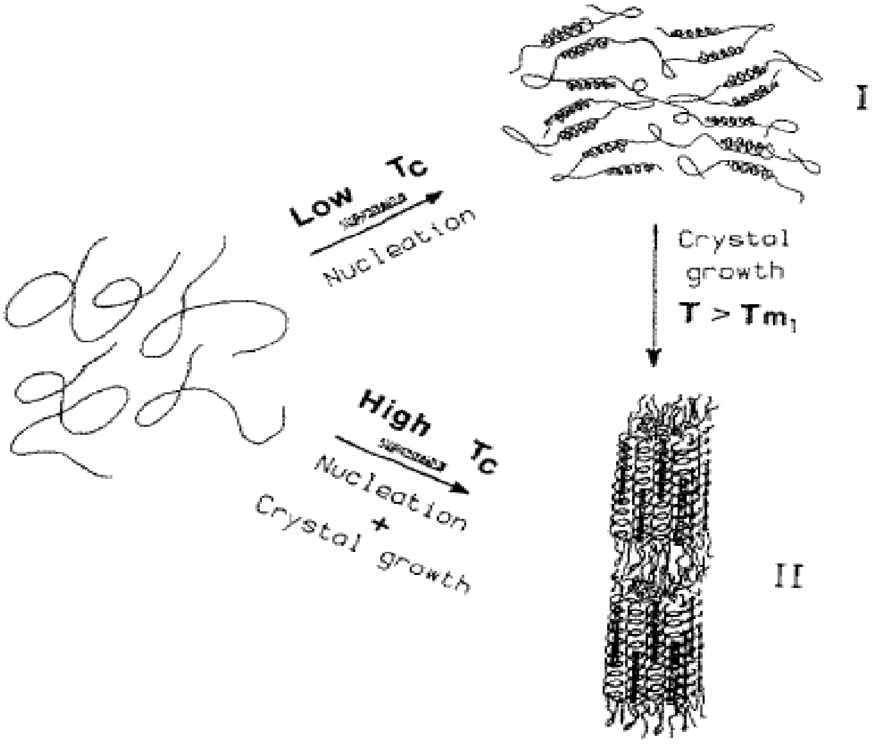


Figure 2.14 Generalized mechanism for amylose-lipid complex formation (Biliaderis & Galloway, 1989)

Type II amylose-stearic acid complexes (formed at 90°C) dissociated at between 114–125°C (Karkalas *et al*., 1995). Only type II complexes exhibit birefringent properties (Biliaderis & Galloway, 1989) and are more crystalline than type I (Tufvesson *et al*., 2003). Type II complexes can be converted from type I complexes through partial melting of the latter (Biliaderis & Galloway, 1989). Type II complexes give a strong wide-angle X-ray diffraction pattern (reviewed by Morrison, 1995). There are two forms of type II complexes. Type IIa are semi-crystalline and formed during gelatinization (as reviewed by Morrison, 1995) or they are fully crystalline and formed after gelatinization (Type IIb). Type IIb complexes are thought to be the most stable form of the amylose-lipid complexes and form ordered crystalline superstructures (Rappenecker & Zugenmaier, 1981). Superstructures are formed when the uncomplexed segments of amylose chains are folded parallel or anti-parallel (Raphaelides & Karkalas, 1988;

Biliaderis and Galloway, 1989). Intramolecular hydrogen bonds stabilize the complexes (Rappenecker & Zugenmaier, 1981). This superstructure formation is facilitated by water molecules between helices form hydrogen bonds with oxygen molecules (two, three, four & six) on the exterior V_H-amylose glycosyl residues (Rappenecker & Zugenmaier, 1981; Karkalas & Raphaelides, 1986).

2.5 Possible roles of crystalline starch structures in food

The increase in the concerns of consumer to lower their fat intake leaves the food producer with the challenge of producing low fat or fat free products with the same level of quality as their full fat counterparts (Aime *et al*., 2001; Muir *et al*., 1999). Modified starches have been applied as fat substitutes in a wide range of food products such as ice cream, feta type cheeses and processed cheese analogues as well as in meat products (Muir *et al*., 1999; Sipahioglu *et al*., 1999; Aime *et al*., 2001 and Hoffman & Mellet, 2003). The starch simulates the functional properties of fat with reduced calorific value (Muir *et al*., 1999).

Small-sized starch particles may be of great use in the food industry because of their novel physical properties (Kim & Lim, 2009). Small-sized starch particles (micron or nano sized) produced through the reaction of starch with fatty acids, may act as fat substitutes in the production of low fat or fat free food products. The small size of the starch particles could possibly enable them to physically mimic the lipid micelles (Dang & Copeland, 2003) while the lipid fraction may also act as carriers of flavour (Tari *et al*., 2003; Singh & Kim, 2009).

Starch-lipid composites (SLC) prepared by steam-jet cooking and added to yoghurt resulted in stronger gels due to possible co-aggregation with casein micelles (Singh & Kim, 2009). The addition of SLC to yoghurt could therefore increase yoghurt viscosity and the fat fraction could act as a flavour carrier as well as improve mouthfeel (reviewed by Singh & Kim, 2009). Other diary products, such as mayonnaise, could also benefit from addition of starch-lipid particles.

CHAPTER 3

HYPOTHESES AND OBJECTIVES

3.1 Hypothesis

The complexation of stearic acid with tef and commercial maize starches, followed by extended cooking in the rapid visco analyzer, may result in the formation of a second viscosity peak as a result of amylose-lipid inclusion complexes formed. The formation of a second viscosity peak during extended cooking was first observed by Nelles *et al*. (2000) for maize starch and later for tef starch by D'Silva (2009). The second peak has been attributed to the formation of amylose-lipid inclusion complexes (Nelles *et al*., 2000). This has however not been proven.

Amylose-lipid complexes formed during extended pasting of starches modified with stearic acid will alter the microstructure of tef and maize starches with stearic acid compared to starches without stearic acid (control). Nelles *et al*. (2003) observed changes in maize starch pastes coinciding with the second viscosity peak breakdown. They reported that granule integrity was lost after second peak formation as amylose-lipid complexes formed (Nelles *et al*., 2003). The amylose-lipid inclusion complexes may alter starch paste structure due to the formation of crystalline particles. Crystalline particles, believed to be amylose-lipid complexes, have been observed by Fanta *et al*. (2002), Byars *et al.* (2003) and Peterson *et al*. (2005) upon cooling of cooked starch pastes.

The modification of tef and maize starches with stearic acid, during extended pasting, will change the starches form crystalline A to amorphous A and V type crystallinity. V type crystalline starches are resultant of the formation of amylose-lipid inclusion complexes (Godet *et al*., 1995; Tang & Copeland, 2007 and D'Silva, 2009).

3.2 Objectives

1. To determine the effect of stearic acid (0.25% and 1.50% on the weight basis of dry starch) on the microstructure of tef and commercial maize starch pastes sampled at six predetermined points during an extended pasting profile (holding period of 2 or 4 hours at 91°C).

2. To determine the effect of stearic acid on the formation of amylose-lipid complexes for tef and commercial maize starches

CHAPTER 4

RESEARCH

The effect of stearic acid on the microstructure and thermal properties of tef and maize starches

ABSTRACT

Naturally occurring fatty acids, for example stearic acid, can modify the pasting properties of starches to produce a large second viscosity peak. The objective of this investigation was to determine the effect of stearic acid (0.25% and 1.50% on the weight basis of dry starch) on the microstructure and thermal properties of tef and maize starches during extended pasting (holding at 91°C for 2 or 4 hours) in the Rapid Visco Analyser (RVA).

Light microscopy and scanning electron microscopy (SEM) of tef and maize starches sampled at six predetermined points during pasting (including first and second peak) and immediately frozen in liquid nitrogen (-180°C), showed increased proportions of intact granules up to the second peak formation (for control and treated samples). DSC (differential scanning calorimetry) of starch pastes modified with 1.50% stearic acid showed endothermic changes in heat flow at temperatures of 99°C-117°C in range with the melting temperatures of amylose-lipid complexes. V_h type crystallinity peaks at 0.44 nm, 0.68 nm and 1.2 nm were observed with X-ray diffraction (XRD) for tef and maize starches modified with stearic acid (0.25% and 1.50%) and sampled at the second peak. The formation of the second peak viscosity during pasting of tef and maize starches modified with stearic acid can be attributed to the formation of amylose-stearic acid complexes.

4.1 Introduction

Fatty acids have been shown to modify starch pasting properties. Kuar & Singh (2000) and Zhou *et al*. (2007) observed reduced peak viscosity, increased time to peak viscosity and increased final viscosity for maize and rice starches containing fatty acids. Reduced peak viscosity and increased time to peak viscosity with the addition of stearic acid to maize starch (Bajner, 2002) and maize and tef starch (D'Silva, 2009) has also been found. It has been hypothized that the lipids form a layer around the starch granule, thereby increasing its hydrophobicity, reducing water uptake and granular breakdown (Richardson *et al*., 2003). The increased final viscosity during pasting may be due to the formation of amylose-lipid complexes (Kuar & Singh, 2000).

Nelles *et al*. (2000) observed the formation of a second peak during extended pasting (longer than 1 hour) of maize starch. The second peak has been proposed to be due to amylose-lipid complex formation, considering the second peak disappeared upon extraction of the fat fraction from the starch (Nelles *et al.*, 2000). Bajner (2002) observed that the addition of up to 1.50% stearic acid increased the second peak viscosity by almost double its size. D'Silva (2009) also observed the second viscosity development in tef and maize starches. When tef starch was pasted with stearic acid no first peak was observed. Although it appears both lipids and amylose play a role in the formation of the second pasting peak, there has been no evidence proving that this peak is due to their interaction to form amylose-lipid complexes. To establish the role of amylose-lipid complexes in the formation of the second peak, further analysis of the starch during this period of pasting is required.

Microscopy, X-ray diffraction (XRD) and differential scanning calorimetry (DSC) analysis have been used in studying starch paste structure (Nelles *et al*., 2003; Fanta *et al*., 2002 and Peterson *et al*., 2005). Nelles *et al*. (2003) observed that maize starch granules (sampled at various times throughout pasting) remained intact until the second peak formation, after which integrity was lost.

Fanta *et al*. (2002) and Peterson *et al*. (2005) used SEM and light microscopy with phase contrast and polarized light optics to show the crystalline spherocrystals formed after steam-jet cooking of maize starch in the presence of lipids. They concluded that these aggregates were amylose-lipid complexes, as the complexes did not form for waxy maize and potato starch. They used X-ray analysis to show that these aggregates showed V-type crystallinity. DSC analysis of the spherocrystals showed melting endotherms similar to those previously reported by Karkalas *et al*. (1995) for amylose-stearic acid complexes (Fanta *et al*., 2002; Peterson *et al*., 2005).

Other than the studies of D'Silva (2009), the effect of fatty acids on the microstructure of tef starch has not been investigated. Compared to maize, tef shows some unusual properties such as lower peak and setback viscosity as well as increased resistance to breakdown during pasting (Bultosa *et al*., 2002). The properties of this underutilized African cereal (tef) needs to be investigated further to discover what unique possibilities it hold for future application.

The objective of this work was to determine the effect of stearic acid on the microstructure and thermal properties of tef and maize starches sampled at various points throughout an extended pasting cycle as well as on the possible formation of amylose-stearic acid complexes.

4.2 Experimental

4.2.1 Materials

Tef grain (*Witkop* variety) was obtained from PANNAR, Kroonstad. South Africa. Commercial maize starch, *Amyral* (from white maize) was obtained from Tonghaat Hullet®, Edenvale, South Africa and was used as reference. Chemicals used were of analytical grade.

4.2.2 Starch extraction

Starch was extracted according to D'Silva (2009). Tef grain was sieved in order to remove any extraneous material. The whole grains were milled in a laboratory hammer mill to pass through an 800 µm screen. The resulting in tef flour was then defatted using hexane in a 1:3 (flour: hexane) ratio for 1 hour at 25°C, while constantly stirring the mixture. After 1 hour the hexane layer was decanted off. The procedure was repeated three times and the flour dried at 25°C in a forced air cabinet. Twenty grams of the defatted tef flour was suspended in 100 ml of distilled water to form a slurry. The slurry was then stirred for 1 hour and passed through a Retsch (Haan, Germany) wet mill with a 250 µm screen. The starch containing liquid was retained while the fibrous residue remaining on the screen was discarded. The liquid was then filtered through first a 75 µm sieve followed by a 38 µm sieve. The residues on the sieves were again discarded. The liquid remaining after sieving was centrifuged at 9940 *g* for 10 min. The supernatant was discarded and the brown layer containing the protein that formed on top of the white starchy layer was scraped off. The pellet obtained was then re-suspended in distilled water and centrifuged again. This procedure was then repeated until no brown protein layer was visible. The tef starch samples were then freeze-dried. The purity of the extracted starch was estimated using light microscopy (results not shown).

To remove endogenous lipids starch samples were defatted according to the method of Nelles *et al.* (2000). Twenty grams of starch was mixed with 150 mL of chloroform: methanol (2:1). The mixture was placed in a shaking water bath at 30ºC for 3 hours. The solvent was then decanted off and the starch was washed with the solvent. The solvent was evaporated and the starch dried overnight in a fume cupboard.

4.2.3 Proximate analysis of extracted tef and commercial maize starches

Moisture, ash and crude fat content were determined according to the Approved Methods: 44-15A, 08-17 and 30-25, respectively of the American Association of Cereal Chemists (AACC, 2000). Moisture was determined by the loss in weight of the samples after drying for 3 hours at 103°C. Ash was determined by difference in sample weight before and after incineration in a muffled oven to burn of all organic matter at 450°C. Crude fat was determined using the Soxhlet method, using petroleum ether (boiling point 40–60°C) to extract the fat. Crude protein (N × 6.25) was determined using a Leco FP528 (Leco Corporation, St.Joseph, Minnesota) according to the Dumas method.

4.2.4 Determination of total starch content of tef and maize starches

The Megazyme total starch assay kit (Megazyme® International Bray, Ireland) was used to determine the starch content. This method uses thermostable α-amylase and amyloglucosidase to hydrolyse starch to glucose. Glucose is then quantified colorimetrically by the glucose oxidase-peroxidase reaction.

4.2.5 Determination of Amylose/Amylopectin of tef and maize starches

The Megazyme amylose/amylopectin assay kit was used to determine the percentage composition of amylose and amylopectin. This method uses the selective quantitative precipitation reaction of concanavalin A (Con A) – a lectin, with amylopectin according to Gibson, Solah & McCleary (1997). Amylose is expressed as a percentage of glucose derived from the hydrolysis of total starch before Con A treatment.

4.2.6 Stearic acid incorporation into tef and commercial maize starches

Stearic acid was added to tef and maize starch in the concentrations 0.25% and 1.50% (on the weight basis of dry starch). Stearic acid was first dissolved in ethanol. The starch was then added to the stearic acid solution and the starch-stearic acid mixture was covered with parafilm and aluminium foil. This was placed in a shaking water bath at 50°C for 30 min. After removal of the parafilm and the foil, the ethanol was evaporated off in a force draught oven at 40°C. The control starch samples were treated exactly the same, but without the addition of stearic acid.

Commercial maize starch was freeze-dried prior to incorporation of the stearic acid in order to reduce its moisture content to that of tef starch.

4.2.7 Starch pasting

A Rapid Visco Analyser Model 3D (Newport Scientific, Warriewood, Australia) was used to conduct the extended pasting profiles. Tef and maize starch (3 g; 14% moisture) controls and complexed with 0.25% and 1.50% stearic acid was suspended in distilled water and the total weight was then adjusted to 28 g. The extended pasting profile began with stirring of 960 rpm at 50ºC for 30 seconds. For the remainder of the cycle stirring was held at 160 rpm. The suspension was heated from 50ºC to 91ºC (heating rate of 5.5ºC/min). For controls and 0.25% stearic acid concentrations, starch suspensions were held at 91ºC for 2 hours 5 min and for 1.50% stearic acid for 4 hours and 30 min at 91ºC before cooling to 50ºC over 10 min (cooling rate of 5.5ºC/min).

Starch suspensions were sampled at various points during pasting. Starch suspensions controls and complexed with stearic acid were sampled at six various points (A - F) (Figure 4.1a). Samples were taken at approximately : A - the beginning of the profile before pasting; B - the first viscosity peak; C - the trough between viscosity peaks; D - the second viscosity peak; E – after the second peak and F - final viscosity/ end of pasting.

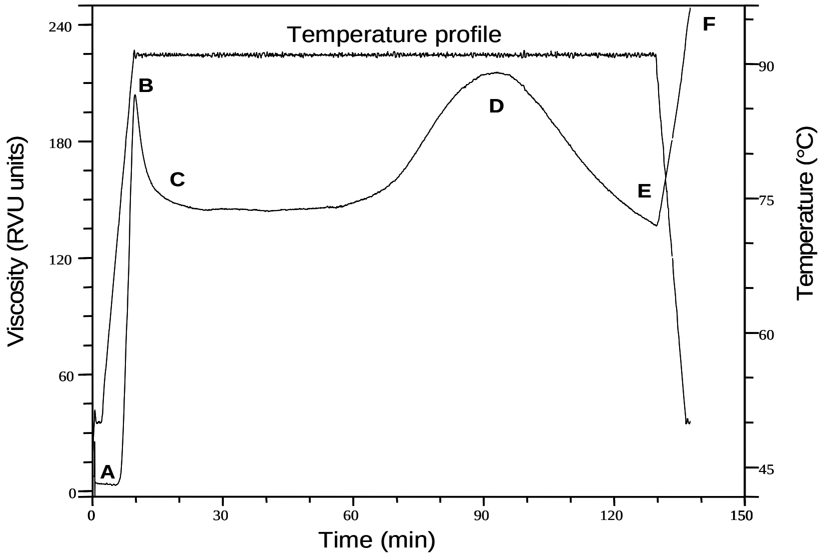


Figure 4.1 Representative biphasic pasting curve indicating the approximate sampling points for tef and maize starches; A–raw starch; B–first peak ; C–trough; D–second peak; E– after second peak and F–final viscosity

Freezing of the starch samples was conducted by use of liquid nitrogen (-180ºC). Starch samples, as sampled above, were immediately transferred from the RVA canister and plunge frozen in liquid nitrogen. Samples were then stored in liquid nitrogen for light microscopy analysis. Selected samples were freeze dried for SEM, XRD and DSC analysis.

4.2.8 Analysis of pasted starch samples

4.2.8.1 Light Microscopy of tef and maize starches

Starch samples (sampled as described in 4.2.7) were prepared for microscopic analysis. The frozen starch sample (about 30 µl) was removed from the cryotube (at room temperature) and placed on a glass slide. Distilled water (3-4 drops) was added and a coverslip was applied. Starch samples were observed using a Nikon Optiphot Transmitted Light Microscope (Tokyo, Japan) with phase contrast optics. Polarized light was used to detect birefringence in the granules. Images captured were representative of what was observed.

4.2.8.2 Scanning electron microscopy of tef and maize starches

Freeze dried starch samples (sampled as described in 4.2.7) were mounted on aluminum stubs covered with double sided tape. The stubs were sputter-coated with gold in a Polaron E 5200 coating unit to a thickness of about ± 20 nm. Prepared samples were than viewed in a JEOL JSM 840 (Tokyo, Japan) SEM operated at 5 kV.

4.2.8.3 Differential Scanning Calorimetry of tef and maize starches

The moisture contents of starch samples were adjusted by vapour phase conditioning prior to DSC analysis. Freeze dried tef and maize starch samples (sampled as in 4.2.7) were placed in a desiccator over a saturated K_2CO_3 solution for five days at room temperature. The moisture contents of the starches were adjusted to 8% (g/100 g dry basis) and determined as in 4.2.3. Thermal properties of freeze dried starch samples were determined using a DSC-Q100 (TA Instruments, Newcastle, Deleware). Approximately 10 mg of tef and maize starch samples (controls and complexed wih 0.25% and 1.50% stearic acid) were weighed into aluminium DSC pans and distilled water (30 mg) was added.

Samples were allowed to equilibrate at room temperature for 2 hours before analyzing in the DSC. Some samples were allowed to equilibrate overnight before being analysed. Sample pans were heated from 30ºC to 140ºC at a rate of 5ºC/min. Onset temperature *(T_o)*, peak temperature *(T_p)*, conclusion temperature (T_c) and enthalpy of gelatinization *(ΔHgel)* were calculated using TA Instruments Universal Analysis 2000.

4.2.8.4 X-Ray diffraction of tef and maize starch

The moisture contents of tef and maize starch samples (controls and complexed with 0.25% and 1.50% stearic acid) were adjusted by vapour phase conditioning prior to XRD as in 4.2.8.3 and moisture content was determined as in 4.2.3 . These were milled using a mortar and pestle. Samples were prepared for X-ray diffraction using the back loading preparation method (where the samples are pressed into the sample holder from the back). The flat surface of the sample on the front is exposed to the X-ray beam. This method minimizes the preferred orientation of crystallites. Samples were analyzed using a PANalytical X'Pert Pro Powder Diffractometer (Ostfildem, Germany) with an X'Celerator detector. The diffractometer was equipped with variable divergence and receiving slits using Fe filtered Co-Kα radiation (1.78901 Å) and operated at 35 kV and 50 mA. Samples were scanned at 25°C with 2θ in the range of 2-90°. Diffractograms were interpreted using X'Pert Highscore Plus software.

4.2.8.5 Statistical Analysis

One-way analysis of variance (ANOVA) was performed to determine differences in the viscosity parameters analysed for tef and maize starch due to added stearic acid (Table 5.2). Means were then compared at $p > 0.05$ using Fischer's Least Significant Difference Test (LSD). The independent variables in the experiment were the concentration of stearic acid and the two starch types.

Values shown are the mean of at least three repetitions. Values within the same row with different letters differ significantly.

4.3 Results and Discussion

4.3.1 Proximate composition of the starches

Table 4.1 shows that the crude fat (ether extract) content of tef starch was similar to that of maize starch. Tef starch and maize starches also had similar amylose contents. Tef starch mean protein content (1.5%) was higher than maize starch (0.7%). This could be due to the different extraction methods used. Extraction of tef starch was performed using distilled water. Metabisulphite (SO_2) is mostly used in extraction of commercial maize starch (Kent & Evers, 1994). Sulphur dioxide (SO_2) solubilises the protein matrix by reducing the disulphide bonds (Bultosa, 2003) therefore protein could have been separated more completely from maize starch.

Table 4.1 Chemical composition of tef and commercial maize starch (g/100 g)

	Tef	**Maize**
Moisture	1.4 (0.1)	0.6 (0.1)
Ash	0.6 (0.0)	0.1 (0.0)
Protein	1.5 (0.1)	0.7 (0.0)
Crude Fat	0.2 (0.1)	0.2 (0.1)
Amylose*	34.6 (0.8)	32.7 (0.7)
Total starch	84.2 (5.7)	89.0 (2.1)

Values in brackets are the standard deviations of the mean
*(g/100 g of total starch)

4.3.2 Pasting properties of tef and maize starches

Figure 4.2 shows the effect of stearic acid (0.25% and 1.50%) on the pasting properties of tef starch. For tef control a first viscosity peak (140 RVU) formed after 10 min, with a breakdown viscosity of approximately 20 RVU (Table 4.2). Stearic acid (0.25% and 1.50%) had an unusual effect on the first viscosity peak of tef starch (Figure 5.1). The addition of 0.25% stearic acid produced no first viscosity peak within the first 15 min of pasting and therefore no time to peak viscosity or breakdown could be calculated. D'Silva (2009) also observed no first viscosity peak for tef starch modified with stearic acid. The addition of 1.50% stearic acid significantly ($p<0.05$) increased the total time to reach a peak viscosity of approximately 100 RVU to 14 min. This viscosity was lower compared to the control. At a concentration of 1.50% stearic acid, breakdown was decreased (compared to control) to approximately 6 RVU. For maize starch (Figure 4.3) the addition of stearic acid (0.25% and 1.50%) did not significantly ($p>0.05$) decrease the first peak viscosity. There appeared to be an increase in time to peak viscosity, but it was not significant ($p>0.05$). There was however, a significant ($p<0.05$) decrease in breakdown viscosity for maize starch with the addition of 1.50% stearic acid.

Decreased peak viscosity, time to first peak viscosity and breakdown was also observed by D'Silva (2009) with the addition of stearic acid to tef and maize starches and also by Zhou *et al*. (2007) with the addition of stearic acid and linoleic acid to rice starch. Bajner (2002) found decreased breakdown after first peak viscosity when stearic acid was added to maize starch at increasing levels and subjected to an extended pasting profile. Zhou *et al*. (2007) explained the effect on peak viscosity, with addition of fatty acids, to be due to the lipids covering the starch granule surface. Richardson *et al*. (2003) suggested that a layer of lipid covering the starch granules would result in increased hydrophobicity of the granules which would lead to decreased water uptake and reduced swelling.

There was no increase in viscosity for tef starch control after the first viscosity peak and all through the holding period at 91°C for 2 hours. At the end of the holding phase upon cooling to 50°C tef starch viscosity increased to final viscosity (193 RVU) (Table 4.2). For maize starch control the formation of a small (159 RVU) second peak, after 70 min during the holding period at 91°C for 2 hours, occurred. Maize starch also increased to final viscosity (206 RVU) upon cooling (Table 4.2). The second pasting peak formed for maize starch control (Fig 4.3 and Table 4.2) could be due to the formation of complexes with endogenous lipids in the starch that were not completely removed by defatting (Nelles *et al*., 2000).

The addition of 0.25% stearic acid to tef (Figure 4.2) and maize (Figure 4.3) starches resulted in the formation of a second viscosity peak (after 70 min), during the holding phase of 91°C for 2 hours. At a concentration of 1.50% stearic acid the second peak viscosity formed after 40 min during holding at 91°C for 4 hours further increased compared to 0.25% stearic acid, for both tef and maize starches. However, the second peak formed for 1.50% stearic acid was noisy, and not a smooth line, as in the case with 0.25%. The reason for the noisy second peak could possibly be due to the limitations of the RVA. The viscosity of the starch (1.50% stearic acid) pastes was possibly at a higher level than the maximum stirring ability of the instrument, making it difficult for the paddle to stir in a smooth continuous manner.

The formation of a second pasting peak viscosity during extended cooking of starch in the RVA was observed for maize starch by Nelles *et al*. (2000) and Bajner (2002) and for tef and maize starches by D’Silva (2009). Nelles *et al*. (2000) showed that upon removal of the lipid fraction the second peak disappeared. D’Silva (2009) pasted waxy maize (97% amylopectin) starch with 0.25% stearic acid and no second peak was formed. The complexation index (CI) also increased with the increasing concentrations of stearic acid added to tef and maize starches possibly indicating less free amylose and more amylose complexed with stearic acid (D'Silva, 2009). The formation of the second peak has been attributed to the formation of amylose-lipid complexes (Nelles *et al*., 2000; Bajner, 2002; Nelles *et al*., 2003 and D'Silva, 2009), as the presence of lipids and amylose appeared to be necessary for the formation of the second peak.

Increasing the stearic acid concentration to 1.50% resulted in an increased area of the second peak for both tef and maize starches compared to the control and 0.25% stearic acid. Increased area of the second peak with increased concentration of stearic acid was also observed for maize starch (Bajner, 2002) and both maize and tef starches (D'Silva, 2009). At a concentration of 1.50% stearic acid a larger amount of amylose-stearic acid complexes, compared to less stable complexes between amylose and endogenous lipids, could be present. This could possibly be the reason for the larger second peak formed with the 1.50% stearic acid treatment. Also, the second peak formed for 1.50% stearic acid, started earlier (after approximately 50 min) than in the case of 0.25% stearic acid (after approximately 70 min). This could be due to the presence of a higher amount of stearic acid and therefore a higher rate of complex formation. The decrease in viscosity following the second viscosity peak could be due to the dissociation of the unstable complexes formed with endogenous lipids (Bajner, 2002).

For both tef and maize starches controls, paste viscosity increased to reach final viscosity, upon cooling. During the cooling phase there is an increase in the viscosity of a starch paste to reach a final viscosity, as a result of the reassociation of the starch molecules in order to re-establish ordered structure that is referred to as retrogradation (Thomas & Atwell, 1999). The addition of 0.25% stearic acid also resulted in the formation of a final viscosity for tef and maize that was higher when compared to the controls. D'Silva (2009) also observed an increase in the final viscosity (compared to the control) for tef and maize starch with the addition of 0.25% stearic acid. She explained that the higher final viscosity of starches modified with 0.25% compared to the control could be as a result of both the formation of amylose-lipid complexes as well as the retrogradation of uncomplexed amylose.

For tef and maize starch modified with 1.50% stearic acid, however, there was no increase in viscosity during the cooling period after second peak formation. It is expected that upon cooling, starch pastes increase in viscosity to reach a final viscosity. However for tef and maize modified with 1.50% stearic acid, the starch pastes decreased in viscosity up to the end of the pasting profile. The observed 'final viscosity' recorded as sampling point F was therefore equal to the lowest viscosity after the second peak formation (sampling point E). Therefore during the cooling period there was probably no retrogradation when tef and maize was modified with 1.50% stearic acid. Thus, addition of increased levels of stearic acid may therefore retard / inhibit retrogradation.

The unpredictably low final viscosity reached during the cooling phase of tef and maize starch pastes modified with 1.50% stearic acid could possibly be due to a larger amount of amylose-stearic acid complexes compared to control and 0.25% stearic acid formed during the second peak. Therefore fewer uncomplexed amylose molecules are available to reassociate and consequently increase the viscosity. The pasting profile used by D'Silva (2009) was too short to observe whether, there was an increase in viscosity during the cooling phase for 1.50% stearic acid.

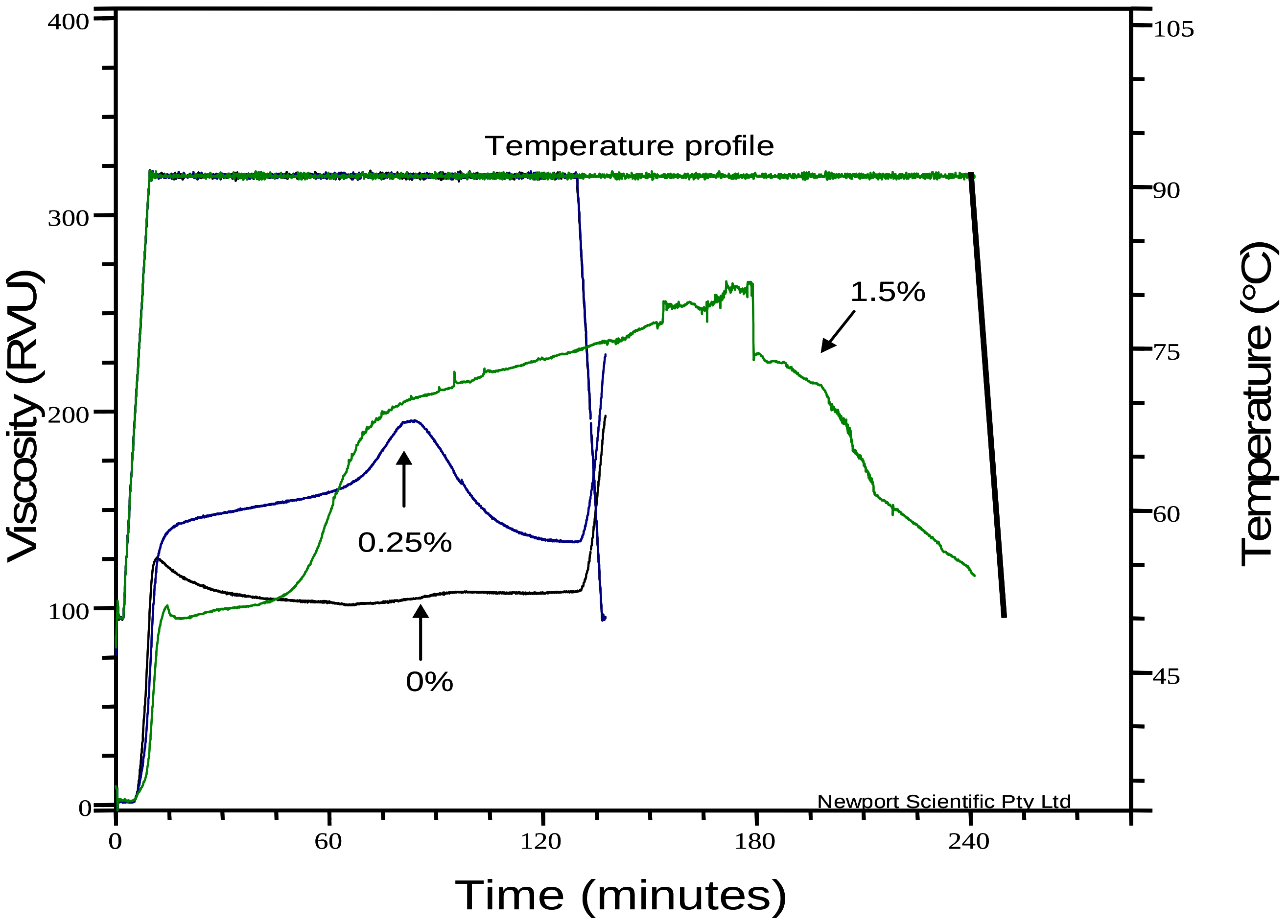


Figure 4.2 Effect of stearic acid addition (0.25% and 1.50%) on the pasting properties of tef starch

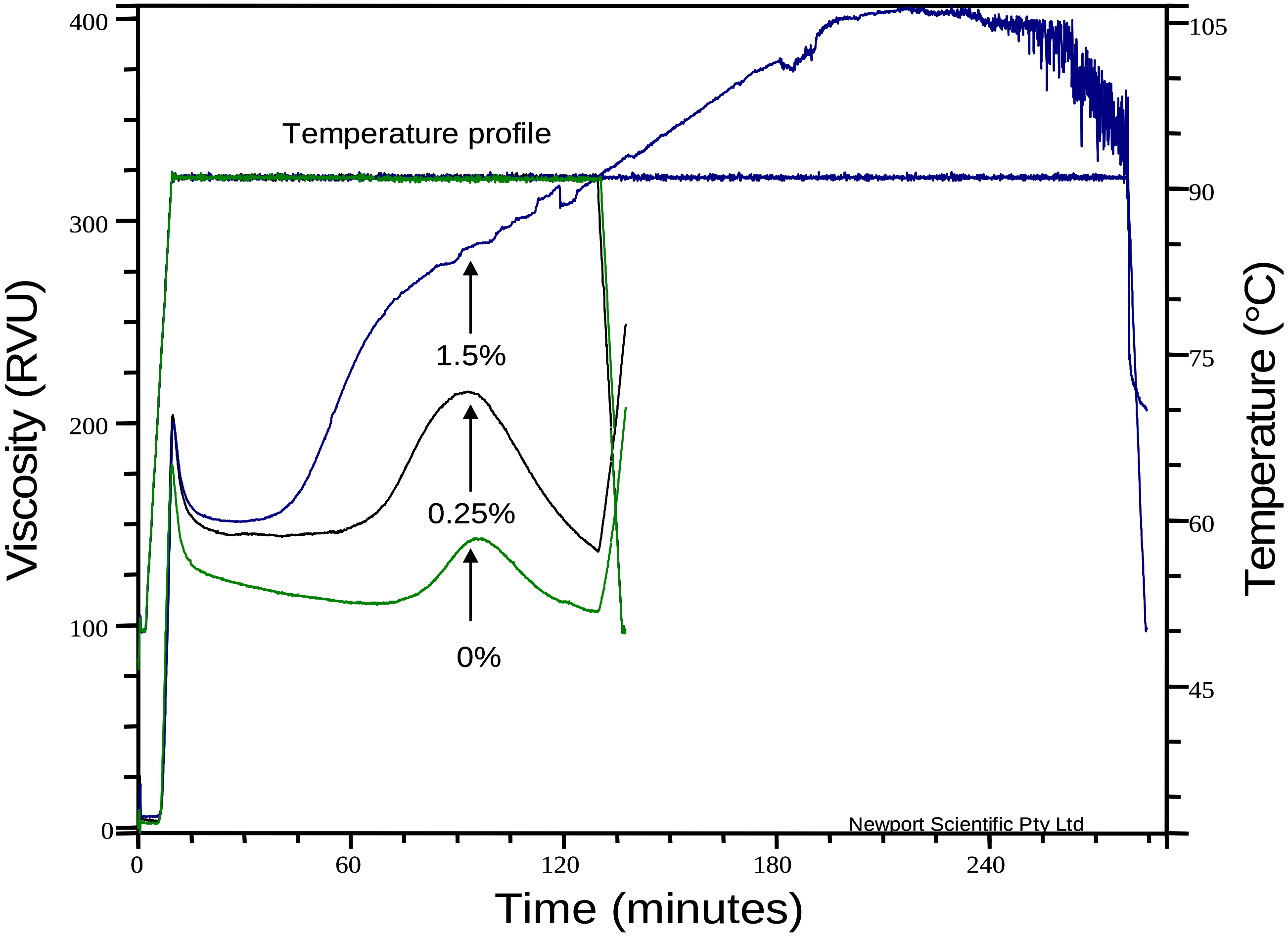


Figure 4.3 Effect of stearic acid addition (0.25% and 1.50%) on the pasting properties of maize starch

Table 4.2 Effects of stearic acid addition (0%, 0.25% and 1.50%) on the viscosity (RVU)# of tef and maize starches sampled at 5 time points (B, C, D, E and F)

Sampling point	Starch	Stearic acid (%)		
		0	0.25	1.50
B (first peak)	Tef	125.5 ^{b} (1.1)	nd*	103.2 ^{a} (3.3)
	Maize	193.29 ^{a} (1.2)	204.9 ^{b} (5.2)	203.5 ^{b}(7.6)
C (trough)	Tef	116.0 ^{b} (1.6)	nd*	94.9 ^{a} (5.6)
	Maize	132.7 ^{a} (6.3)	150.2 ^{b} (0.9)	156.8 ^{b} (1.5)
D (2nd peak)	Tef	nd**	184.7 ^{a} (8.5)	281.4 ^{b} (25.0)
	Maize	158.8 ^{a} (7.8)	213.8 ^{b} (8.1)	372.4 ^{c} (41.5)
E (2nd trough)	Tef	nd**	136.9 ^{b} (2.9)	106.3 ^{a} (9.1)***
	Maize	124.0 ^{a} (3.3)	154.0 ^{b} (9.1)	219.7 ^{c} (19.7)***
F (final viscosity)	Tef	193.1 ^{a} (4.3)	223.7 ^{b} (4.6)	106.3 ^{ab} (9.1)***
	Maize	205.5 ^{a} (4.8)	240.2 ^{b} (9.9)	219.7 ^{ab} (19.7)***

values in brackets are standard deviations from the mean

* Not determined as there was no first peak for tef starch with 0.25% stearic acid

** Not determined as tef starch did not show a first viscosity peak for 0% stearic acid

***In the case of starch modified with 1.50% stearic acid, sampling point E=F, seeing as the pasting profile ended at sampling point E

Mean values in the same rows with the different letters differ significantly *($p<0.05$)*

4.3.3 Microscopy of tef and maize starches

Figure 4.4a and b shows the scanning electron micrographs (SEM) of raw tef and maize starch granules. Individual raw tef starch granules (Figure 4.4a) were polygonal in shape and between 2–6 µm in diameter. The surface of the tef granules appears to be smooth and there was no evidence of surface pores. Bultosa (2003) also did not find any pores on the surface of tef starch using SEM. The surface of maize starch granules (Figure 4.4b), however, contained visible pores. Fannon *et al*. (1992) found pores on the surface of maize starch granules. Raw maize starch granules were found to be between 12–15 µm in diameter. These results are in agreement with those previously reported by Bultosa (2003). When viewed under polarized light (Figure 4.4c and d), raw tef and maize starch granules exhibited birefringence in the form of Maltese crosses.

Figures 4.5, 4.7 and 4.8 respectively show light, phase contrast and SEM micrographs of tef and maize starches, (control and modified with 0.25% and 1.50% stearic acid) sampled at the various points (A–raw starch; B–first peak; C–trough; D–second peak and E/F–final viscosity) during pasting. The granules of tef starch control sampled before pasting (point A) (Figures 4.5a, 4.7a and 4.8a) were intact and not swollen with an average granule size of approximately 5 µm. Similarly, the granules of maize starch control sampled before pasting (point A) (Figures 4.5b, 4.7b and 4.8b) were intact, not swollen and on average approximately 12-15 µm in diameter. Stearic acid modification (0.25% and 1.50%) did not bring about any visible difference in the raw granular structures of tef and maize starch sampled before pasting (point A). Viewing the raw tef and maize starch granules (sampled before pasting) under polarized light showed maltese crosses, indicating these granules were birefringent (Figures 4.4c and d) for both the control as well as the stearic acid modified starches. Complexation with stearic acid did therefore not affect the raw starch crystalline structure. This is because both tef and maize starch controls and modified with stearic acid, still showed birefringence in the form of Maltese crosses.

Light microscopy of tef starch control sampled at the first peak (point B) (Figures 4.5a and 4.7a) showed mostly intact, swollen granules approximately 10-12 µm in diameter. Maize starch control sampled at the first peak (Figures 4.5b and 4.7b) also showed generally intact, swollen granules (25-30 µm in diameter). Some maize starch granules were broken with leached granular contents visible. SEM of both tef (Figure 4.8a) and maize (Figure 4.8b) starch sampled at the first peak (point B) supported the light micrographs by showing intact granules, but it was evident that there were much fewer intact granules for maize compared to tef starch, with large amounts of broken granules present. Additionally, SEM more clearly showed leached out granular content. The considerable swelling of the tef and maize starch granules, due to water uptake during heating, along with leaching out of some granular content is believed to be responsible for the increase in viscosity to reach peak viscosity (Gunaratne & Corke, 2004). Nelles *et al.* (2003) also observed intact maize starch granules up to first peak formation with only some granular content leaching and total loss of birefringence after 10 min of pasting. When viewed under polarized light, tef and maize starch sampled at the first peak did not show any birefringence, indicating that all the granules have gelatinized at this point during pasting.

Considering the light and SEM microscopy data, the addition of stearic acid (0.25% and 1.50%) to tef and maize starch sampled at the first peak (point B) appeared to increase the proportions of intact granules compared to the controls. However, there seemed to be no effect on granular swelling and rupture (compared to the control samples) with the increasing levels of stearic acid. Stearic acid treated tef and maize starch granules were approximately similar in size to the control and granular breakdown and amylose leaching was minimal for all samples.

Richardson *et al*. (2003) observed restricted granule swelling and amylose leakage up to 90°C of wheat starch suspensions modified with a mixture of polyglycerol ester and a monoglyceride (PG/ME) and heated using a Brabender

Viscoamylograph. They suggested that restriction of swelling could possibly be due to lipids covering the granular surface. Reduced amylose leakage was possibly due to the formation of amylose-lipid complexes. Therefore the increasing proportions of intact granules with addition of stearic acid could be due to more lipids being available to form a layer around tef and maize starch granules and thereby retarding their breakdown. Regarding granule swelling, it is important to note that starch samples were taken at the maximum height of the first peak and not at certain predetermined time point. The possibility that stearic acid restricted granule swelling and amylose leaching of tef and maize starch granules should not be disregarded, as there was an increase in time to reach peak viscosity (see section 4.3.2). Restricted granular swelling might have been noted more easily if samples were taken at corresponding time/temperature points corresponded, instead of sampling at a certain point on the pasting curve.

Light microscopy of tef starch control sampled at the trough (point C) (Figures 4.5a and 4.7a) mostly showed the presence of intact swollen (15-20 µm) granules. Some broken granules were also visible. The intact granules seemed to clump and form masses of intact granules. Additionally, SEM (Figure 4.8a) showed leached out granule contents (probably amylose) that could be seen as a network between and surrounding the intact granules. Light micrographs of the maize starch control (point C) showed some intact as well as broken granules with granular fragments and leached out granular content (Figures 4.5b and 4.7b).

It appears from the light microscopy of tef starch (sampled at C) with stearic acid (0.25% and 1.50%) that the proportions of intact granules did not appear to change nor affect the leaching out of their contents. However, due to the large amounts of small sized tef granules it was difficult to clearly distinguish swollen intact granules from leached out or broken granules using light microscopy. Additionally, SEM seemed to indicate larger proportions of intact tef granules (Figure 4.8a). The addition of stearic acid (0.25% and 1.50%) to maize starch (sampled after the first peak) (Figures 4.5b, 4.7b and 4.8b) seemed to lead to increased proportions of intact, swollen granules (25–30 µm) compared to the control, but similar to samples taken at the second peak, the proportions of intact granules were much less compared to tef starch. The granules of both tef and maize starch (control, 0.25% and 1.50% stearic acid) sampled at the trough appeared to aggregate and form larger clumps of multiple granules and there were fewer proportions of individual granules.

As mentioned, a hydrophobic layer of stearic acid around tef and maize starch granules might have retarded water uptake and consequently swelling, leading to larger amounts of intact granules compared to the control. Nelles *et al*. (2003) also observed intact granules up to second peak development and attributed this to be a result of amylose interacting with lipids to form amylose-lipid complexes.

The light micrographs of tef (Figures 4.5a and 4.7a) and maize (Figures 4.5b and 4.7b) starch controls sampled at the second peak (point D) showed lower proportions of intact swollen granules and larger proportions of pasted starch networks, when compared to samples taken at previous points (A, B and C). This was similar to what Nelles *et al*. (2003) found. They suggested that the second peak formation coincided with granular breakdown, as they also observed granular breakdown of most intact granules at this point. Formation of the second viscosity peak has been attributed to the formation of amylose-lipid inclusion complexes (Nelles *et al*., 2000). No complexes were, however, observed by these authors.

There seemed to be no difference with the addition of 0.25% stearic acid to tef and maize starch compared to the control samples. For 1.50% stearic acid, however, light (phase contrast) and SEM micrographs of both tef (Figures 4.7a and 4.8a) and maize (Figures 4.7b and 4.8b) starches sampled at the second peak (point D), showed larger proportions of intact granules, than the controls. In the case of tef starch granules, without surface pores, the high concentration of stearic acid (1.50%) could cause more granules to be layered with the hydrophobic fatty acid, as suggested by Richardson *et al*. (2003), thereby retarding granule breakdown. D'Silva (2009) also observed that stearic acid formed a layer around tef starch granules. For maize starch granules (with surface pores) stearic acid that possibly diffused into the granules could have complexed with amylose, resulting in the formation of amylose-lipid complexes. Stearic acid has been observed to be inside maize starch granules (D' Silva, 2009). This would also lead to reduced swelling and possibly hinder granular breakdown. Possible amylose-stearic acid complexes were observed when maize starch modified with 1.50% stearic acid (sampled at the second peak) was viewed under polarized light (Figure 4.6a). Bright spherical structures (10-20 µm in diameter) exhibiting birefringence were observed for maize starch. When viewed under normal light these structures appeared smaller and darker in colour compared to the surrounding intact maize starch granules. They also seemed to have a dark layer on the surface. Under polarized light, the structures resembled those observed by Fanta *et al*. (2002) prepared jet cooking suspensions of maize, rice and high amylose starch. However, very few of these structures were observed throughout all the microscopic investigations (in total only about eight of these structures were observed) and they were very difficult to find. Additionally, none of these structures were visible when using SEM.

Fanta *et al*. (2002) and Peterson *et al*. (2005) observed the formation of birefringent spherocrystals upon cooling of jet-cooked maize starch. The spherocrystals were not formed from waxy maize, defatted maize or potato starches and were therefore suggested to be amylose-lipid inclusion complexes. D'Silva (2009) also observed small bright particles for tef and maize starches modified with 0.25% stearic acid. The structures observed by D'Silva (2009), however, did not resemble those found in this study and were possibly uncomplexed aggregates of residual stearic acid. Zabar *et al.,* (2009) prepared amylose-stearic acid complexes from amylose and 7% (w/w) stearic acid and used SEM to investigate their microstructure. Amylose-stearic acid complexes were embedded in the surrounding matrix and were between 2–6 µm in diameter.

Because the structures observed for maize starch modified with 1.50% stearic (Figure 4.6) acid were observed for samples taken at the second peak and these structures showed birefringence (Maltese crosses) under polarized light and also resembled the structures observed by Fanta *et al*. (2002) they are hypothesised to be amylose-lipid complexes. This along with the very large area of the pasting peak (Figure 4.3) (see section 4.3.2) and the large V_h- type diffraction peaks for maize starch sampled at the second peak (Figures 4.20) (see section 4.3.5 later), further supports the theory that the observed structures would be amylose-stearic acid complexes. However, none of these birefringent structures were observed for tef starch modified with stearic acid (1.50%).

Another possibility, however, not supporting the hypothesis, is that these birefringent structures observed for maize starch could have been maize starch granules that during heating dried on the surface, thereby forming a hard crust. The granules would not be able to then absorb water and gelatinize. These ungelatinized maize starch granules would then remain birefringent throughout heating.

Tef and maize starch controls sampled after the second peak (point E) and at the end of pasting (point F) were observed to be mostly a homogenous mass with very few intact granules surrounding the starch gel network. SEM of tef starch (Figure 4.8a) sampled after the second peak (point E) also showed the presence of intact tef granules for the control sample. Light microscopy (Figures 4.5a and 4.7a) of the tef starch sampled at points E and F indicated that the gel network seemed to consist of individual swollen granules, as the outlines of the granules were still visible. The gel networks of maize starch consisted of a homogenous mass that remained similar in structure to the final viscosity (F) (Figures 4.5b and 4.7b).

The addition of stearic acid (0.25% and 1.50%) to tef starch sampled after the second peak (E and F) did not seem to have any visible effect, when compared to the control (Figures 5.4a and 5.6a). For maize starch sampled after the second peak (E/F), the addition of stearic acid (1.50%) seemed to result in intact swollen granules outside of the gelled starch network (Figures 5.4b and 5.6b).

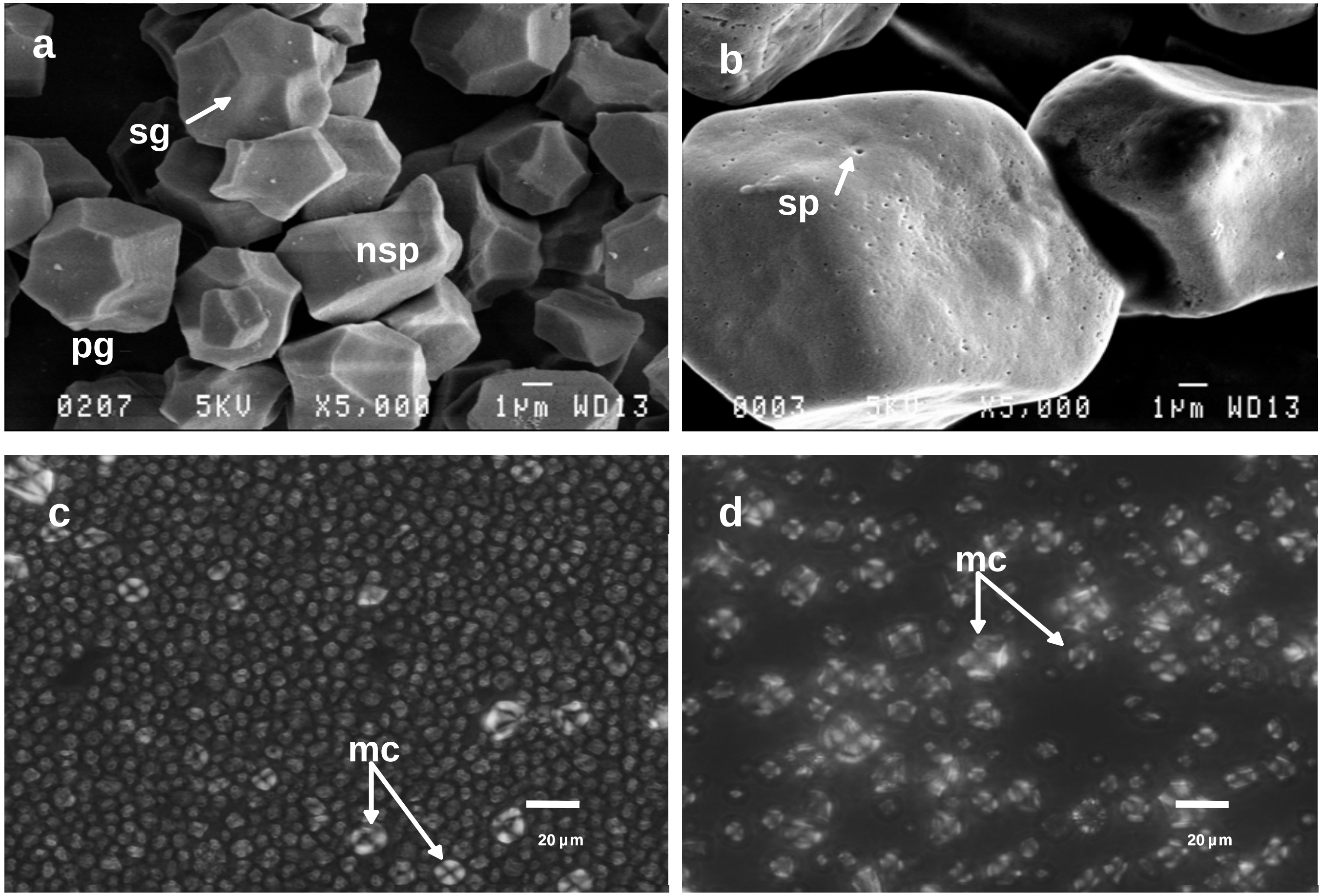


Figure 4.4 Scanning electron micrographs (a & b) and polarised light micrographs (c & d) of tef (a & c) and maize (b & d) starch granules, (sg, starch granules; nsp, no surface pores; sp, surface pores; pg, polygonal and mc, maltese crosses)

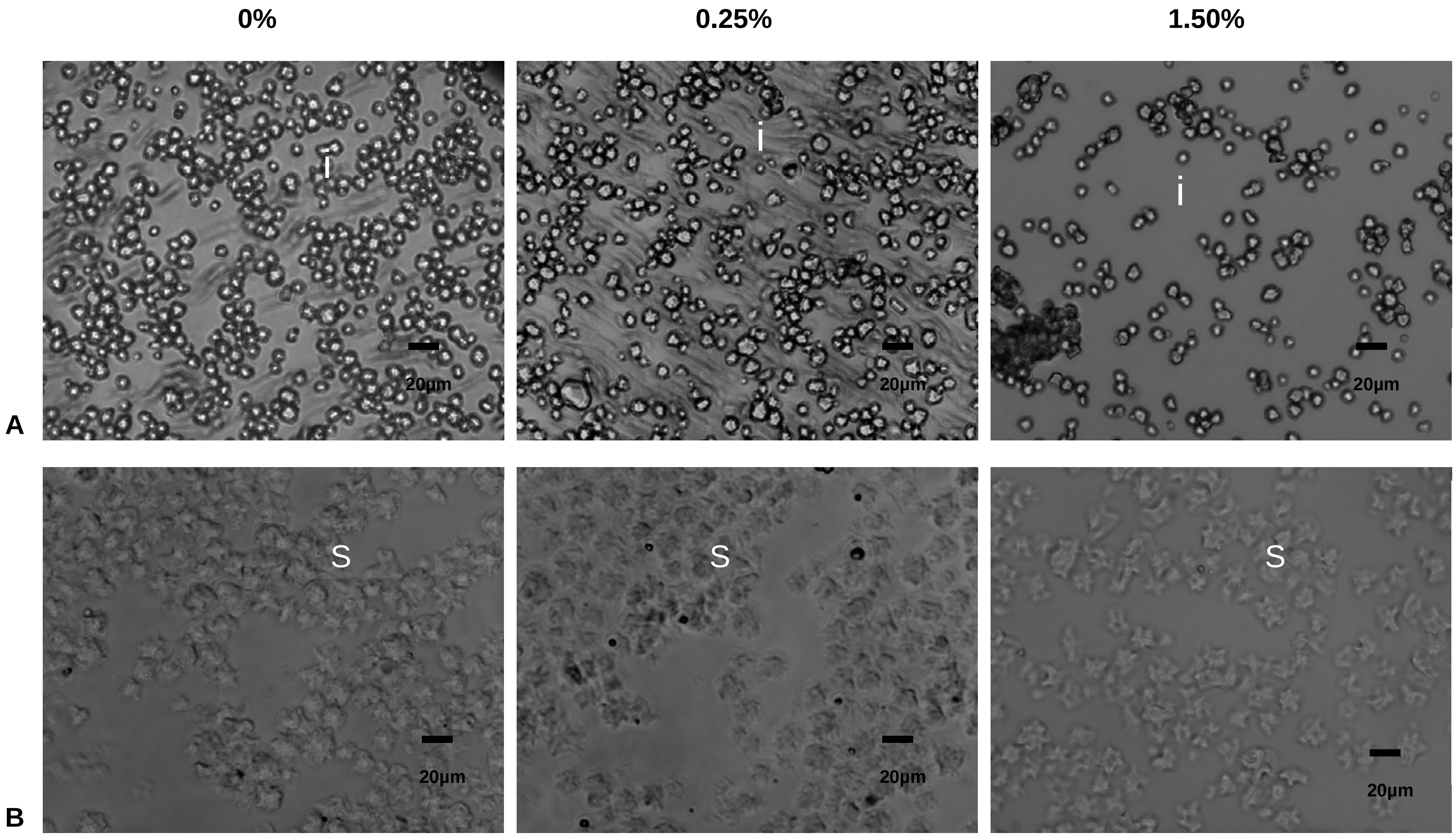


Figure 4.5 (a) Light micrographs showing the effect of stearic acid addition (0.25% and 1.50%) on tef starch sampled at A – 0 min , B – 10 min/1st peak, during pasting. S, swollen starch granules; I, intact raw starch granules

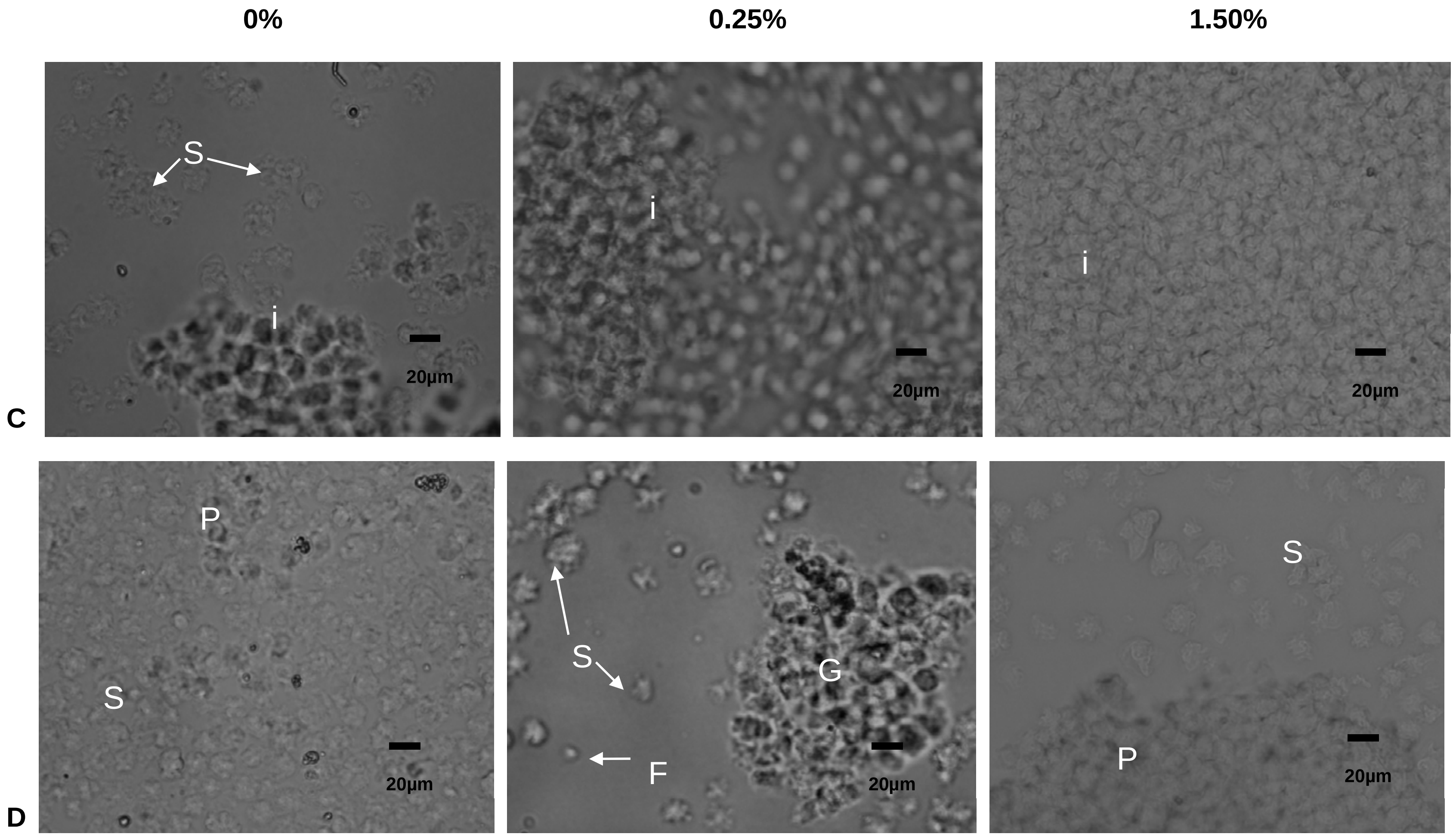


Figure 4.5 (a) (cont) Light micrographs showing the effect of stearic acid addition (0.25% and 1.50%) on tef starch sampled at C – trough; D – 2nd peak during pasting. S, swollen starch granules; I, intact granules; P, pasted starch ; F, stearic acid

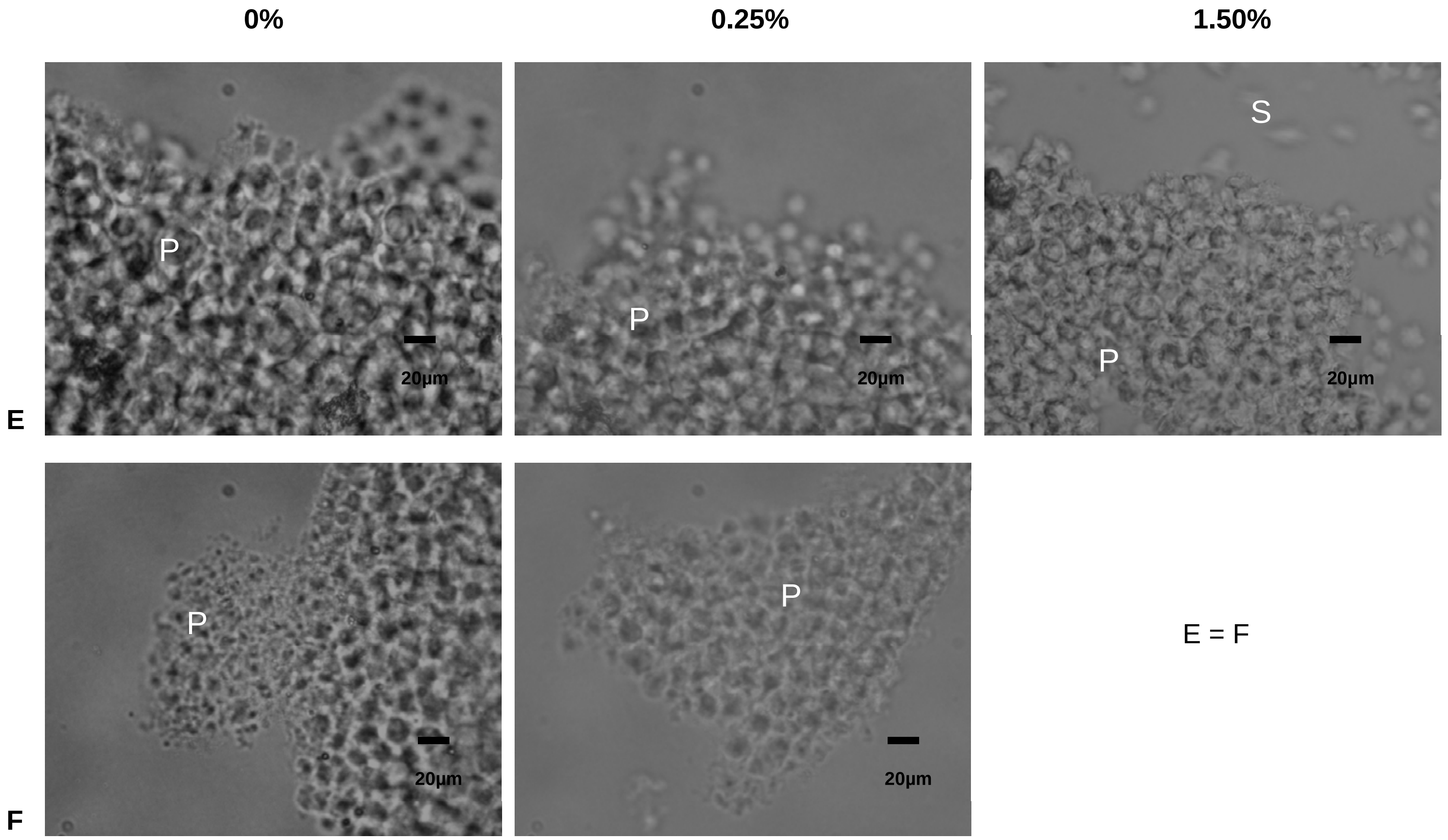


Figure 4.5 (a)(cont) Light micrographs showing the effect of stearic acid addition (0.25% and 1.50%) on tef starch sampled at E – after 2nd peak & F – final viscosity, during pasting. S, swollen starch granules; P, pasted starch.

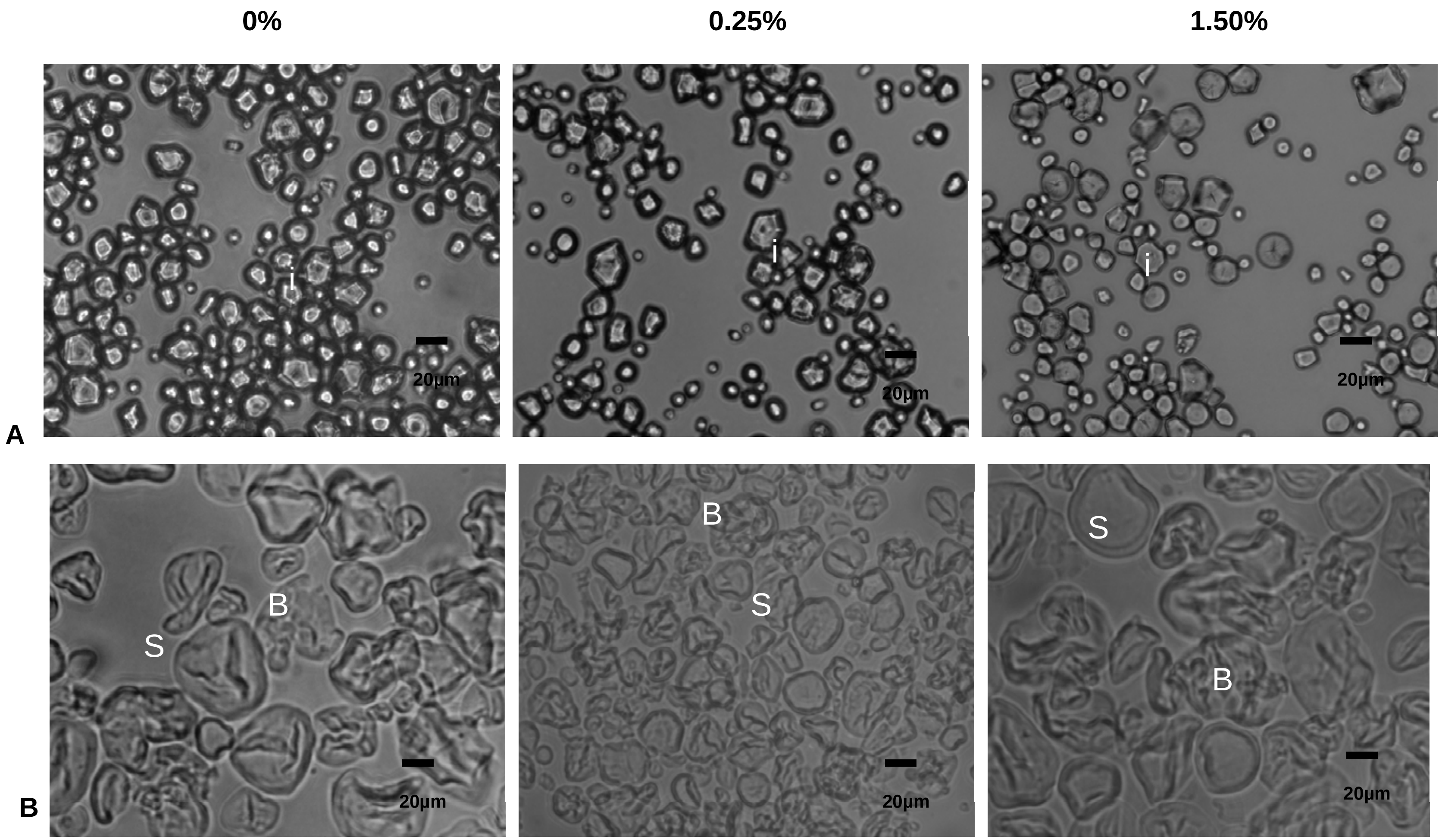


Figure 4.5 (b) Light micrographs showing the effect of stearic acid addition (0.25% and 1.50%) on maize starch sampled at A – 0 min & B – 1st peak, during pasting. S, swollen starch granules; I, intact raw starch granules; B, broken starch granules

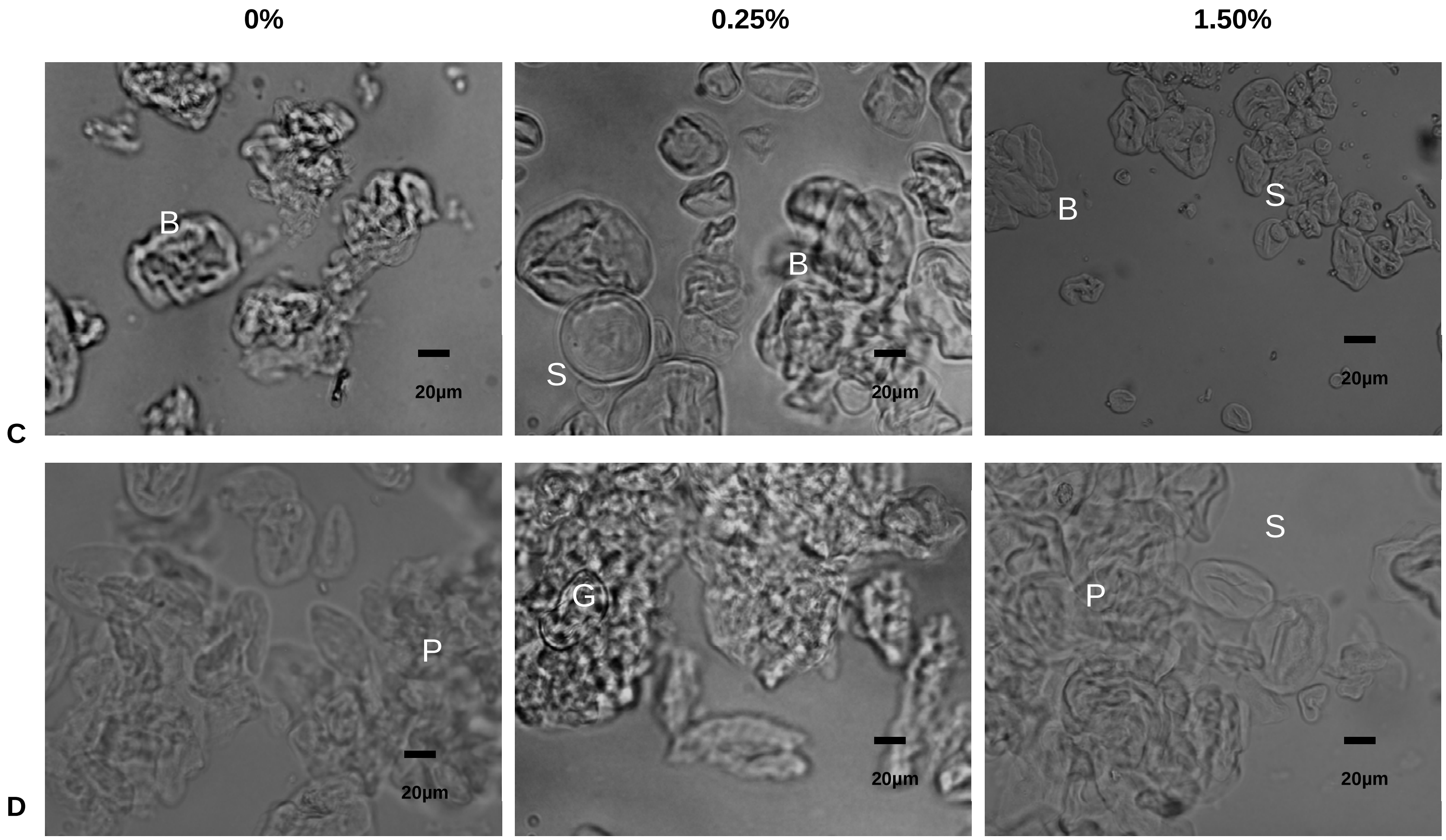


Figure 4.5 (b) (cont) Light micrographs showing the effect of stearic acid addition (0.25% & 1.50%) on maize starch sampled at C – trough; D – 2nd peak, during pasting. S, swollen starch granules; B, broken granules; P, pasted starch

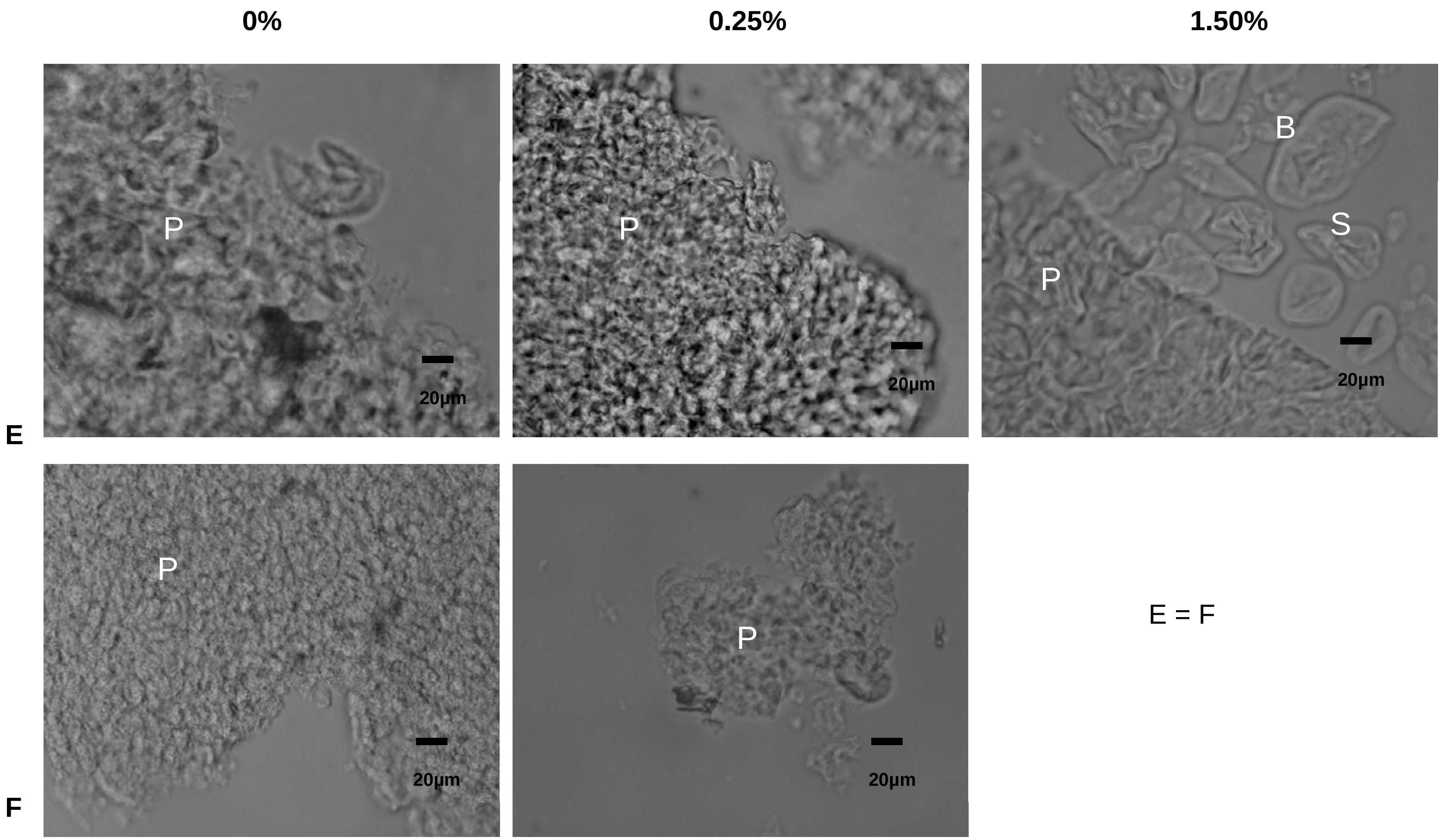


Figure 4.5 (b) (cont) Light micrographs showing the effect of stearic acid addition (0.25% & 1.50%) on maize starch sampled at E – after 2nd peak & F – final viscosity, during pasting. B, broken granules; S, swollen starch granules & P, pasted starch.

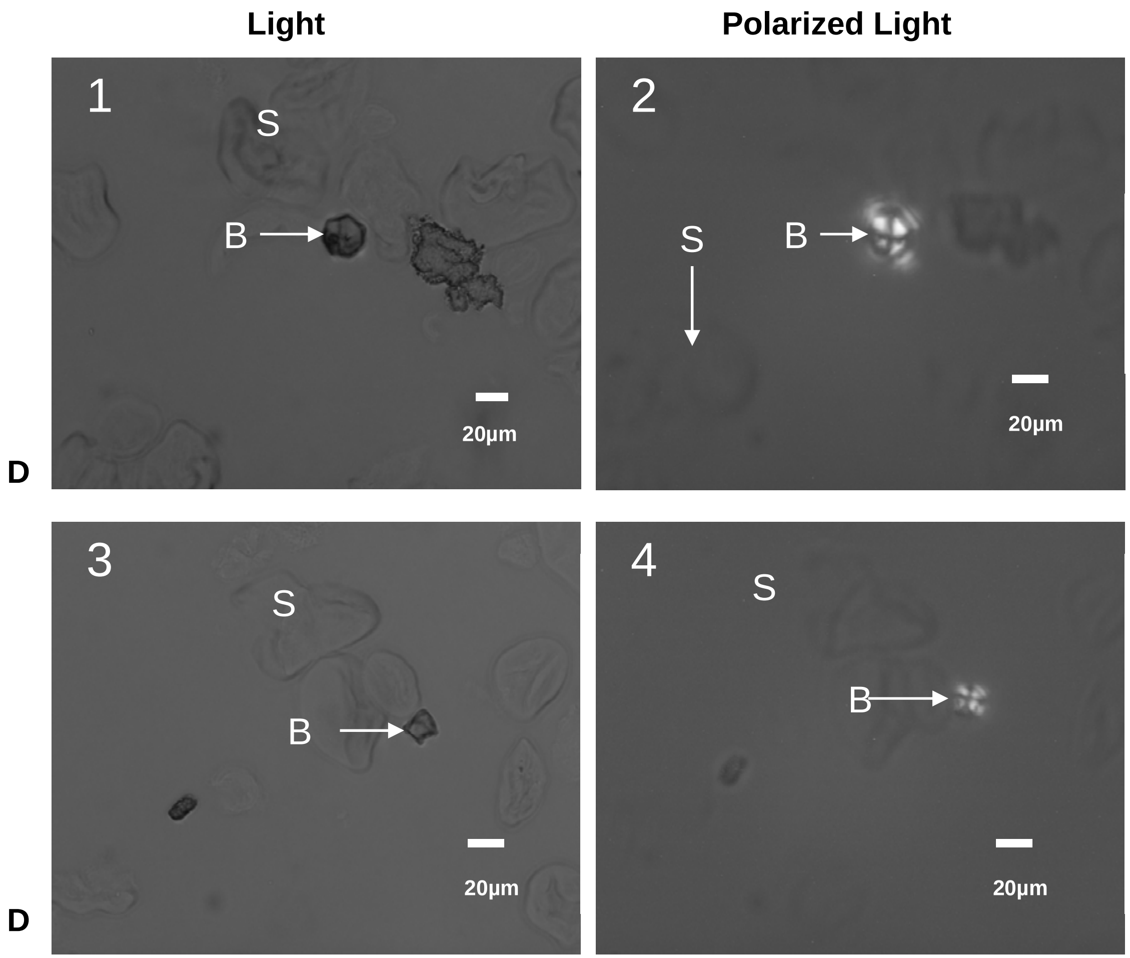


Figure 4.6 (a) Light micrographs of maize starch treated with 1.50% stearic acid sampled at D - 2nd peak, Light micrographs (1 and 3) and polarized light micrographs (2 and 4). B, birefringent structures

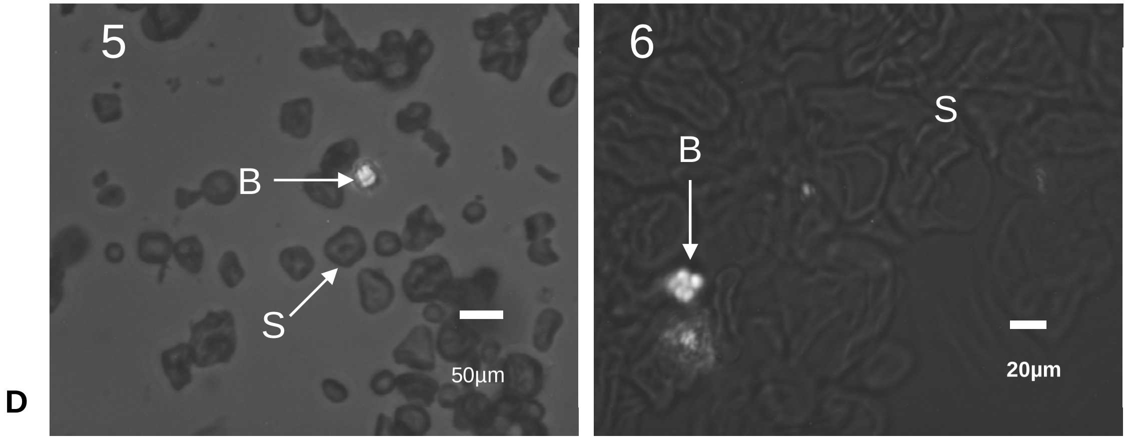


Figure 4.6 (b) Light micrographs of maize starch treated with 1.50% stearic acid sampled at D - 2nd peak, B, birefringent structures; S, starch

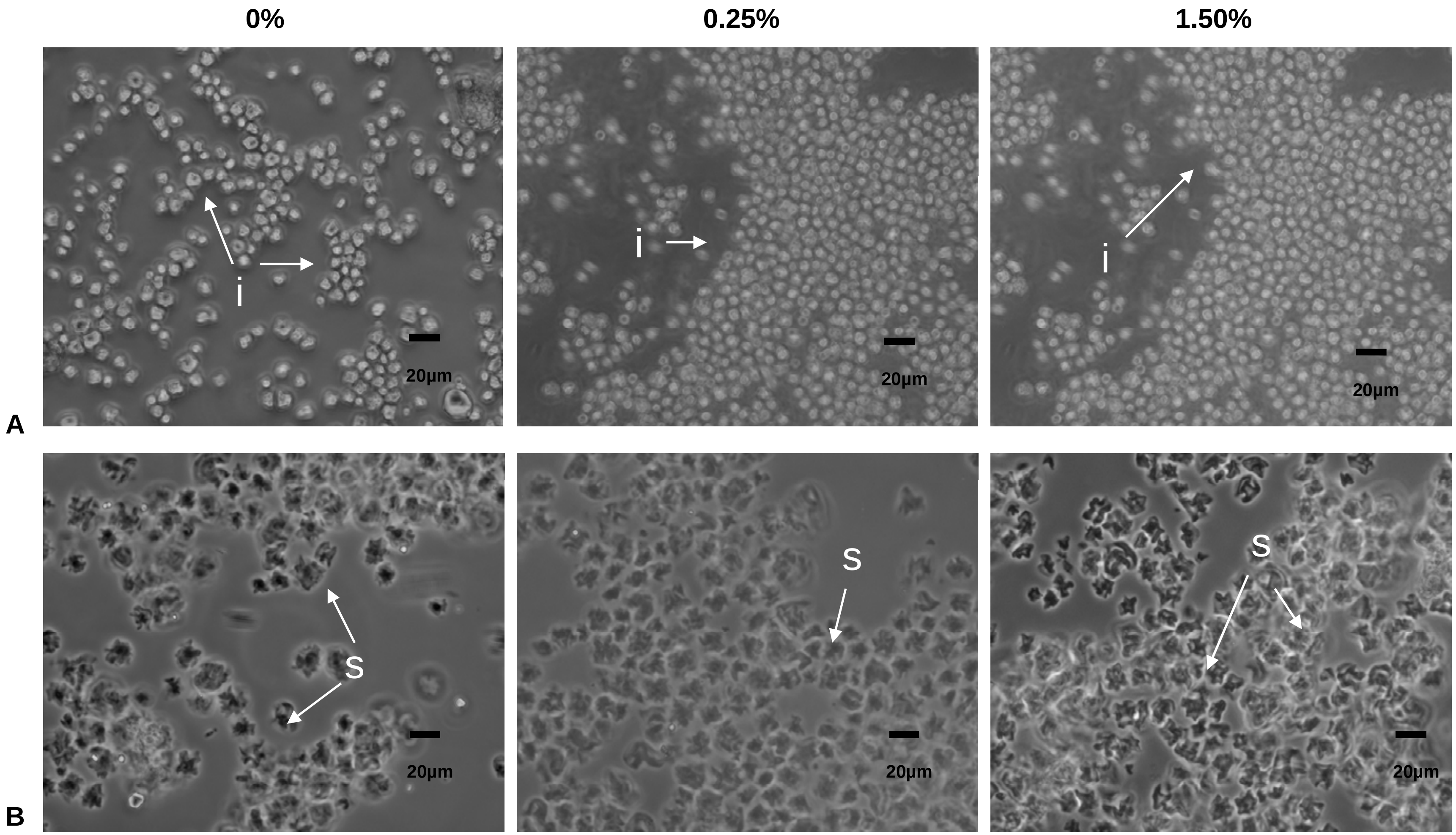


Figure 4.7 (a) Phase contrast light micrographs showing the effect of stearic acid addition (0.25% and 1.50%) on tef starch sampled at A – 0 min & B – 1st peak, during pasting. S, swollen starch granules; I, intact raw starch granules

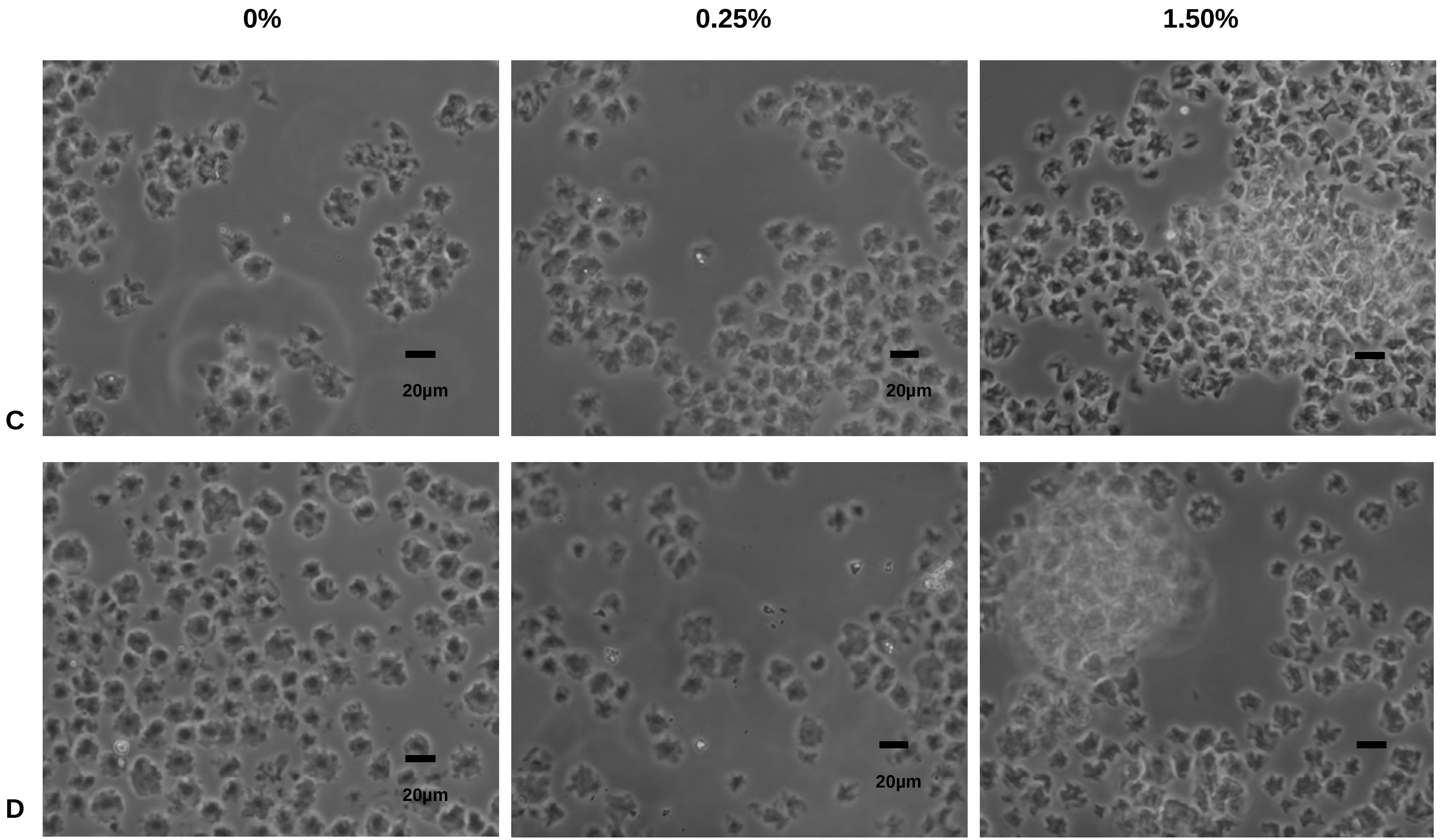


Figure 4.7 (a)(cont) Phase contrast light micrographs showing the effect of stearic acid addition (0.25% & 1.5%) on tef starch sampled at C – trough; D – 2nd peak, during pasting

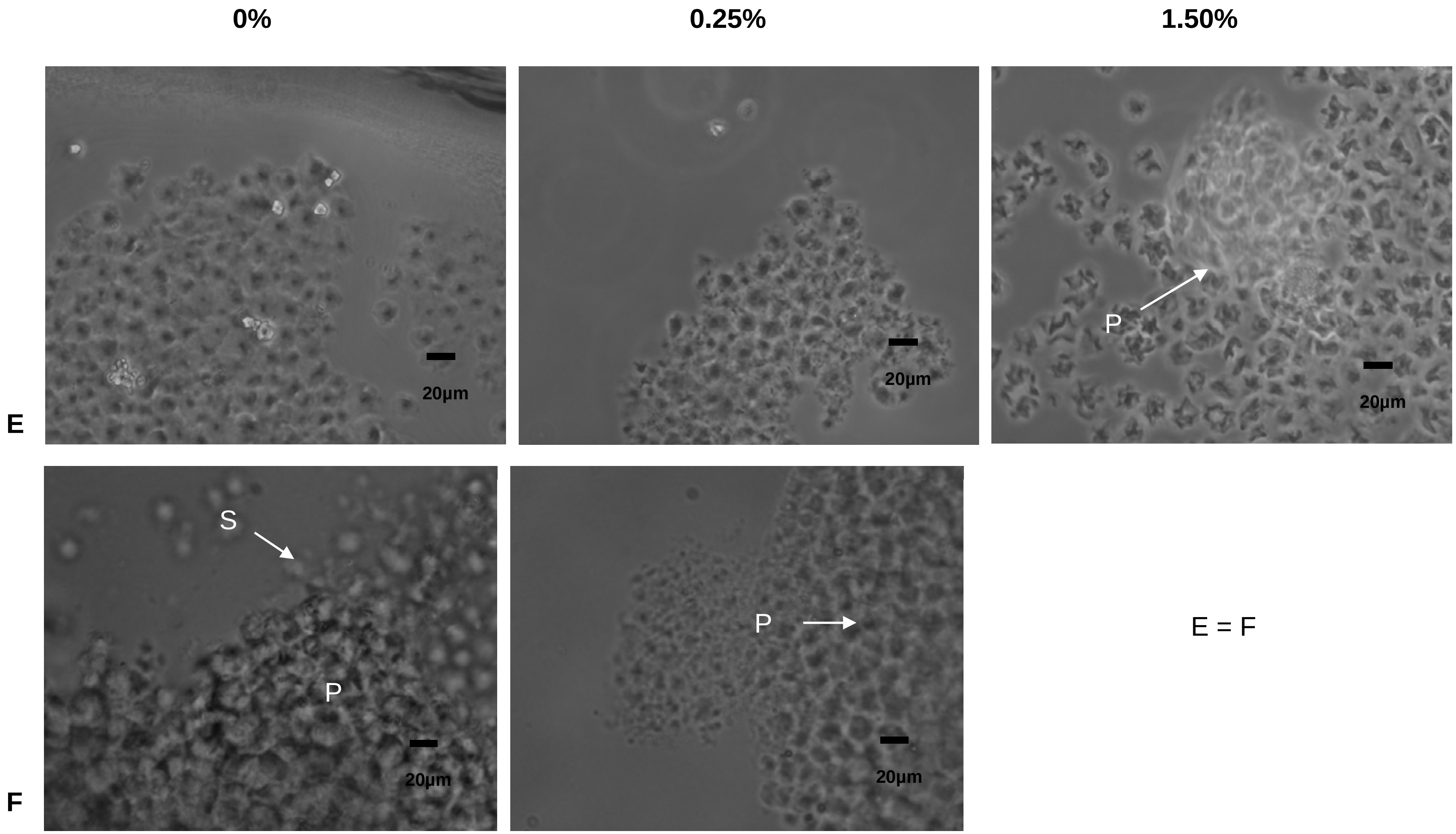


Figure 4.7(a)(cont) Phase contrast light micrographs showing the effect of stearic acid addition (0.25% & 1.50%) on tef starch sampled; E – after 2nd peak& F – final viscosity, during pasting. P, pasted starch

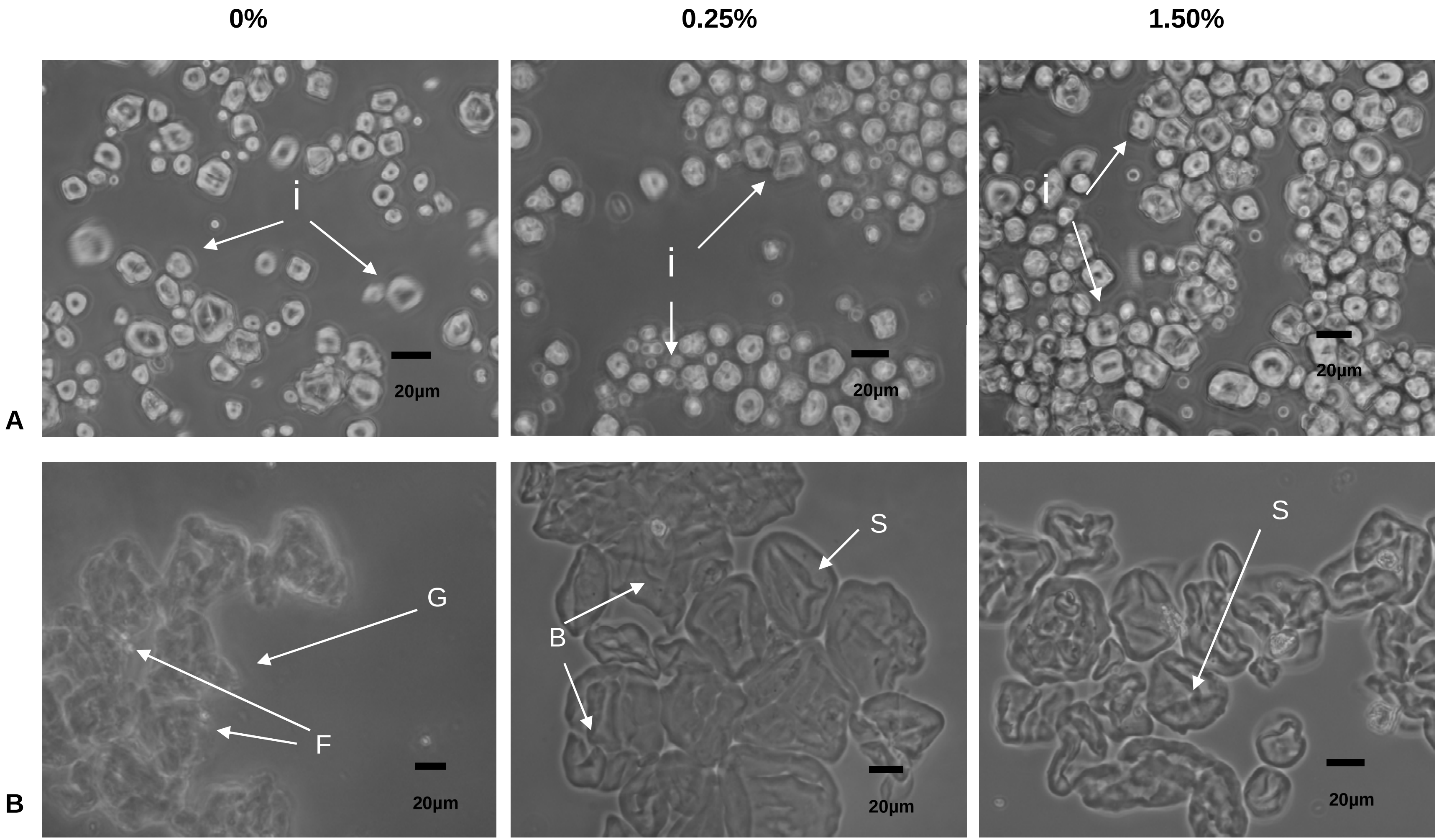


Figure 4.7 (b) Phase contrast light micrographs showing the effect of stearic acid addition (0.25% and 1.50%) on maize starch sampled at A – 0 min & B – 1st peak, during pasting. S, swollen starch granules; I, intact raw starch granules; B, broken granules; F, stearic acid

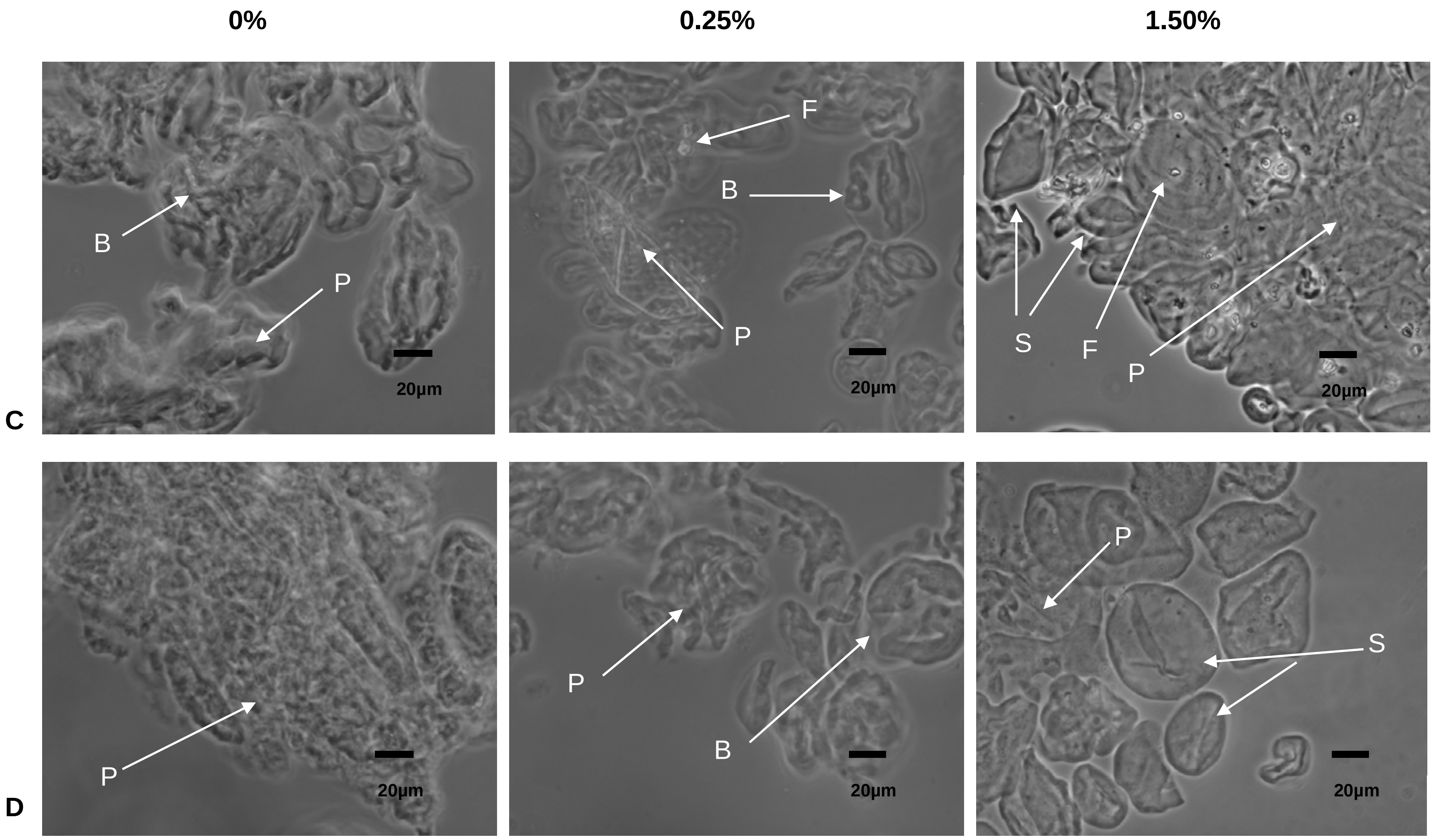


Figure 4.7 (b) (cont) Phase contrast light micrographs showing the effect of stearic acid addition (0.25% & 1.50%) on the of maize starch sampled at C – trough; D – second peak, during pasting. S, swollen starch granules; I, intact raw starch granules; B, broken granules; F, stearic acid; P, pasted starch

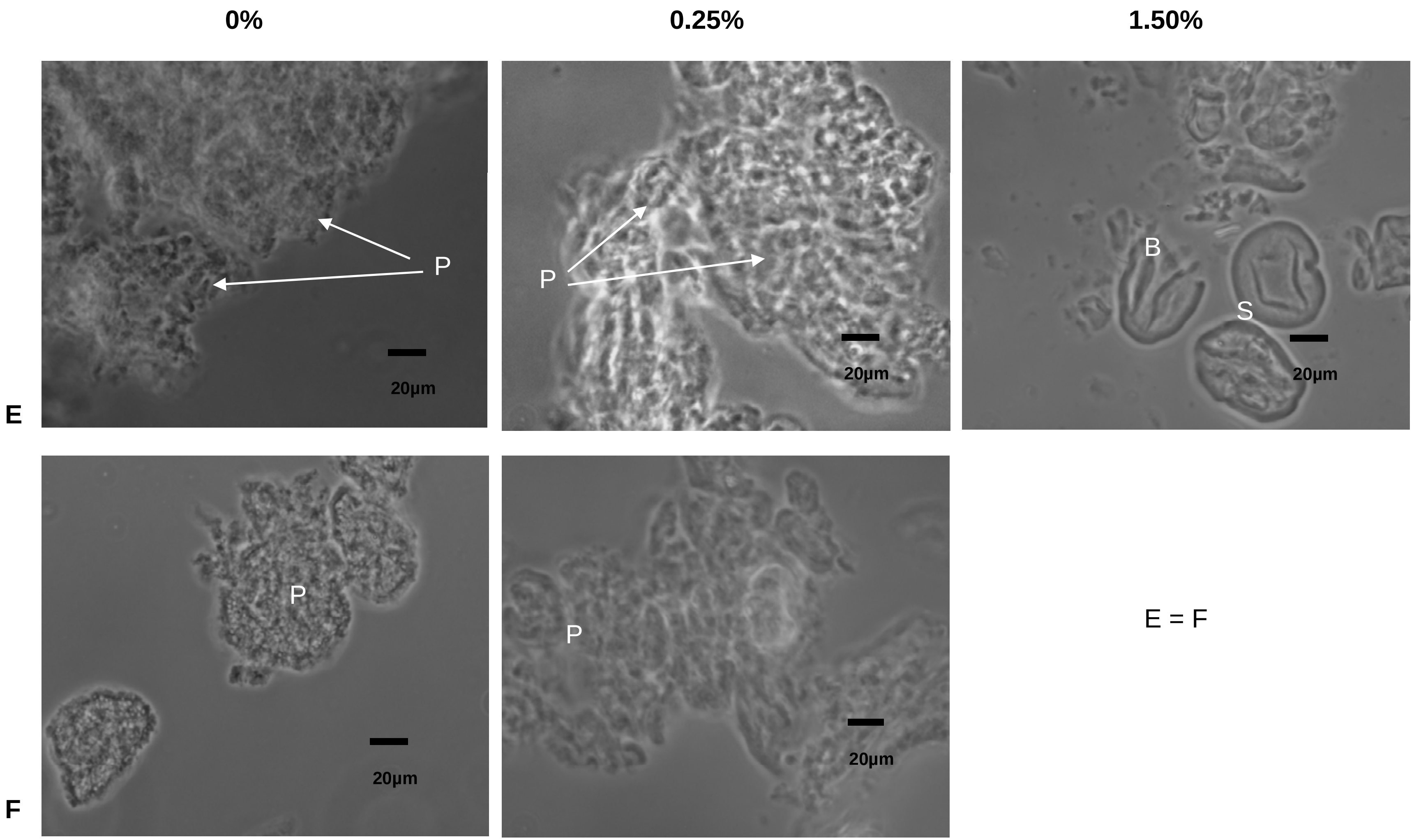


Figure 4.7 (b) (cont) Phase contrast light micrographs showing the effect of stearic acid addition (0.25% and 1.5%) on maize starch sampled at E – after 2nd peak & F - final viscosity, during pasting. S, swollen starch granules; P, pasted starch

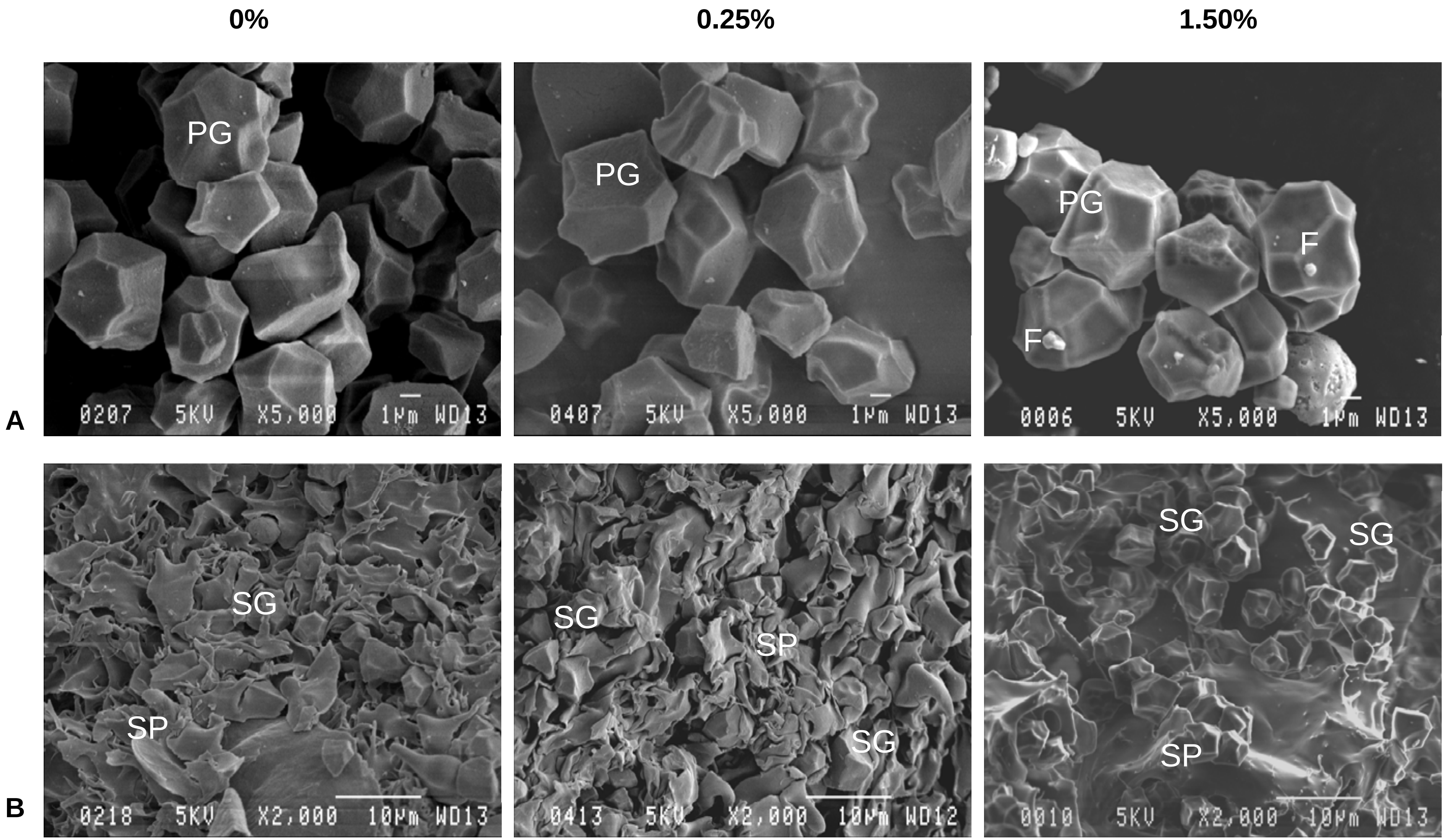


Figure 4.8 (a) Scanning electron micrographs showing the effect of stearic acid addition (0.25% and 1.5%) on tef starch sampled at A – 0 min & B – 1st peak, during pasting. SG, swollen starch granules; PG, polygonal raw starch granule; SP, starch paste

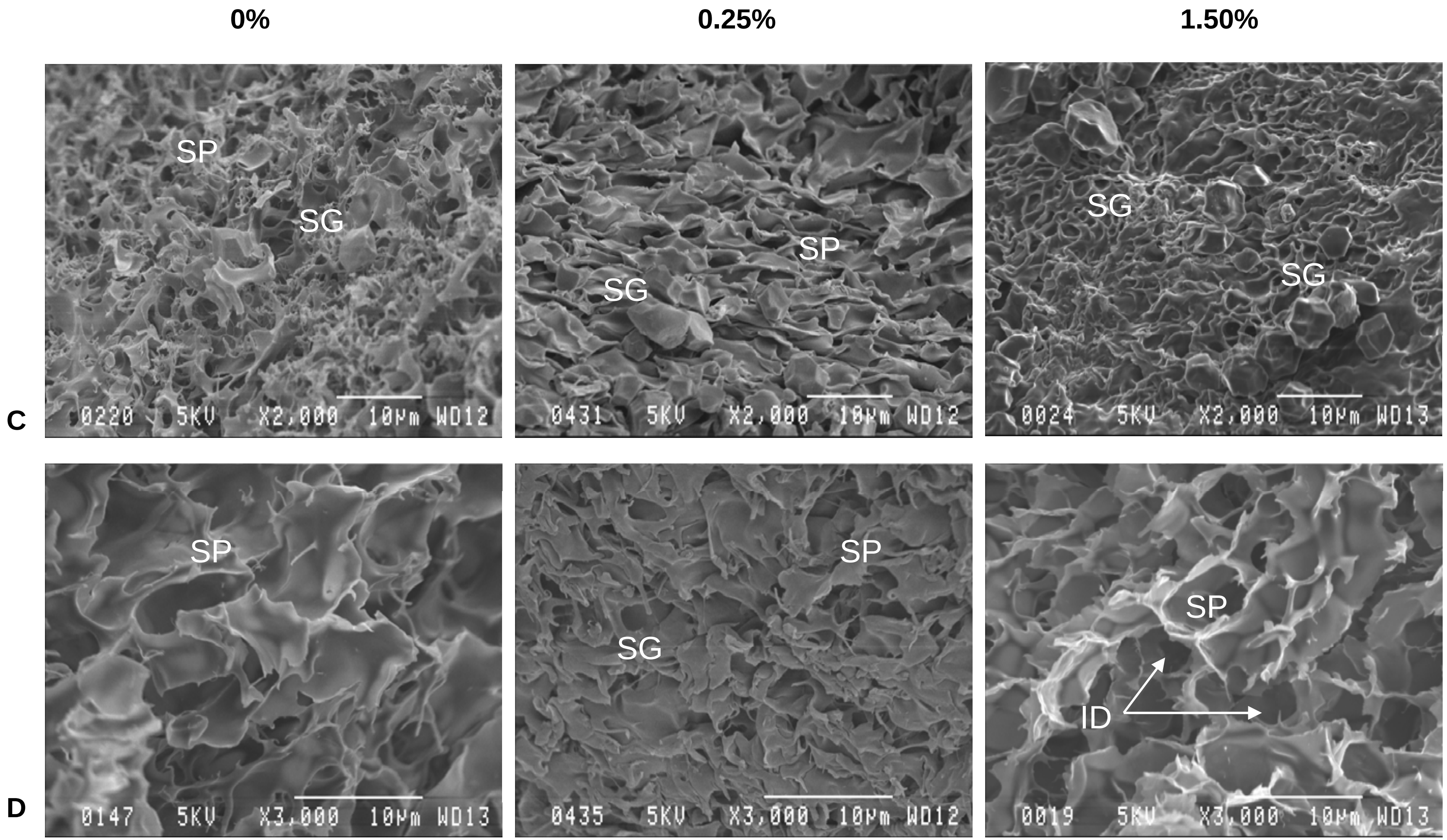


Figure 4.8(a) (cont) Scanning electron micrographs showing the effect of stearic acid addition (0.25% & 1.50&) on tef starch sampled at C – trough; D – 2nd peak, during pasting. SG, swollen starch granules; SP, starch paste and ID, ice crystal damage

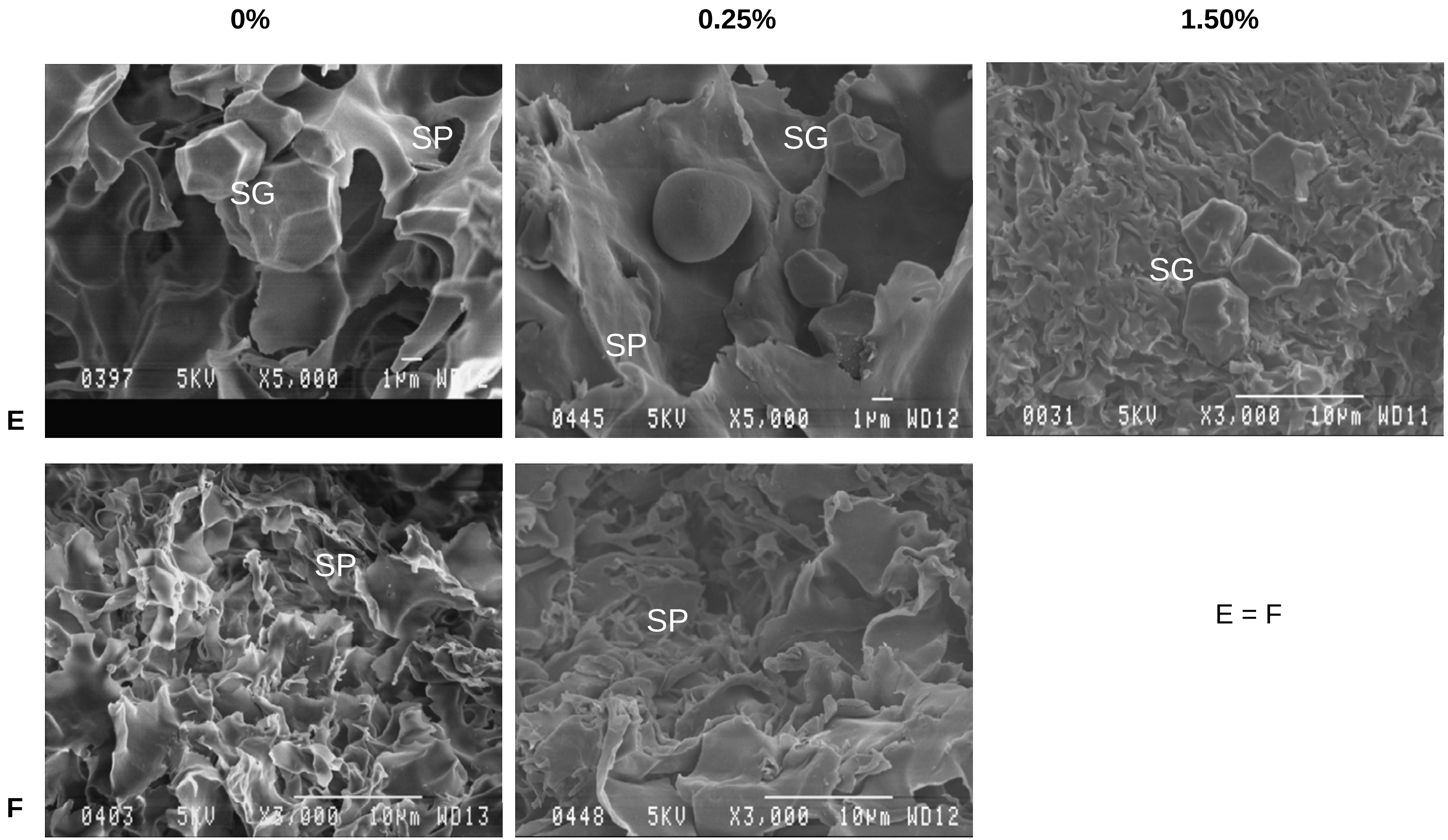


Figure 4.8 (a) (cont) Scanning electron micrographs showing the effect of stearic acid addition (0.25% and 1.50%) on tef starch sampled at E- after second peak; F – end of pasting, SP, starch paste; SG, starch granules

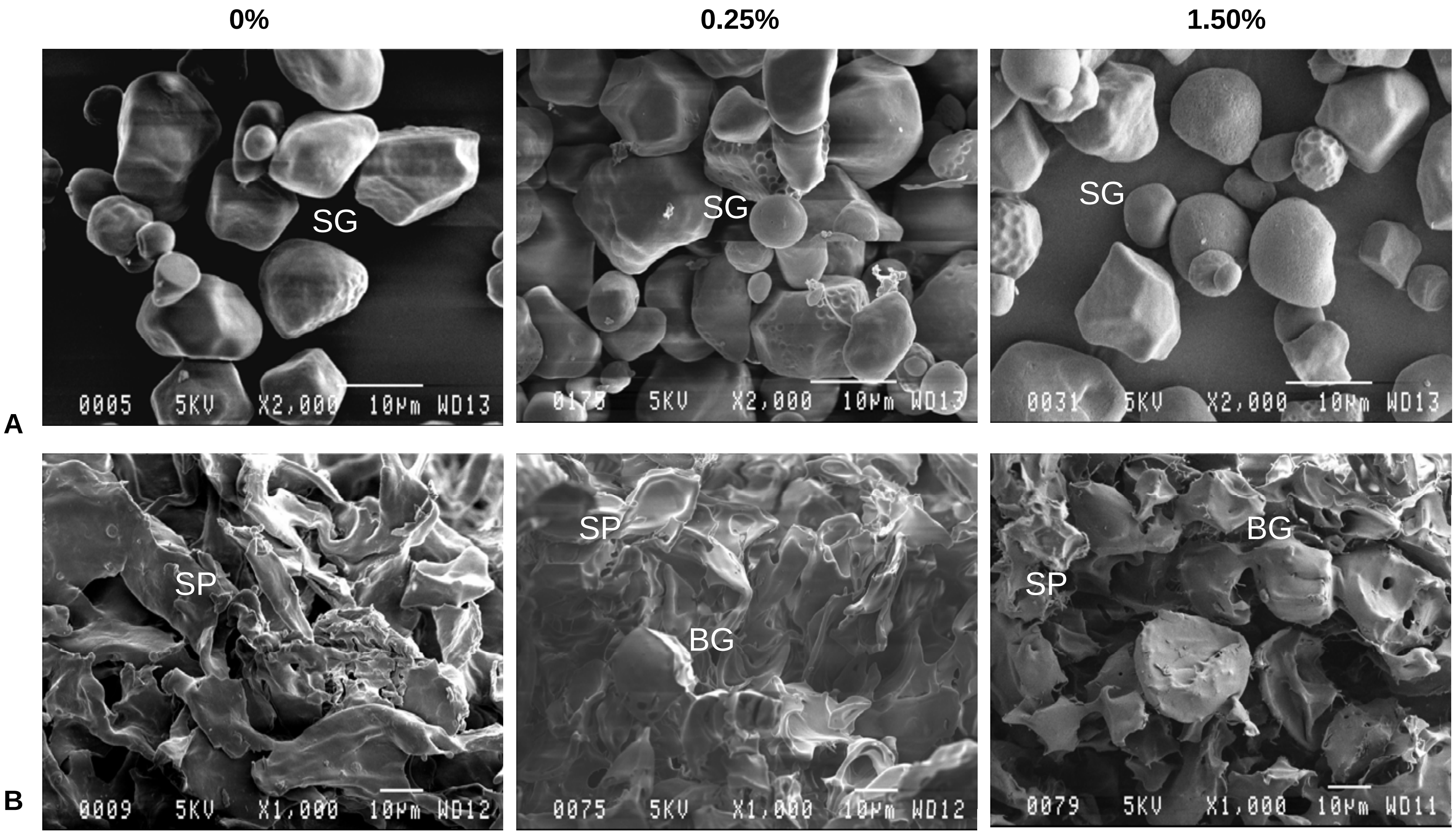


Figure 4.8 (b) Scanning electron micrographs showing the effect of stearic acid addition (0.25% and 1.50%) on maize starch sampled at A – before pasting and B – first peak, SG, raw starch granules ; SP, starch paste; BG, broken granules

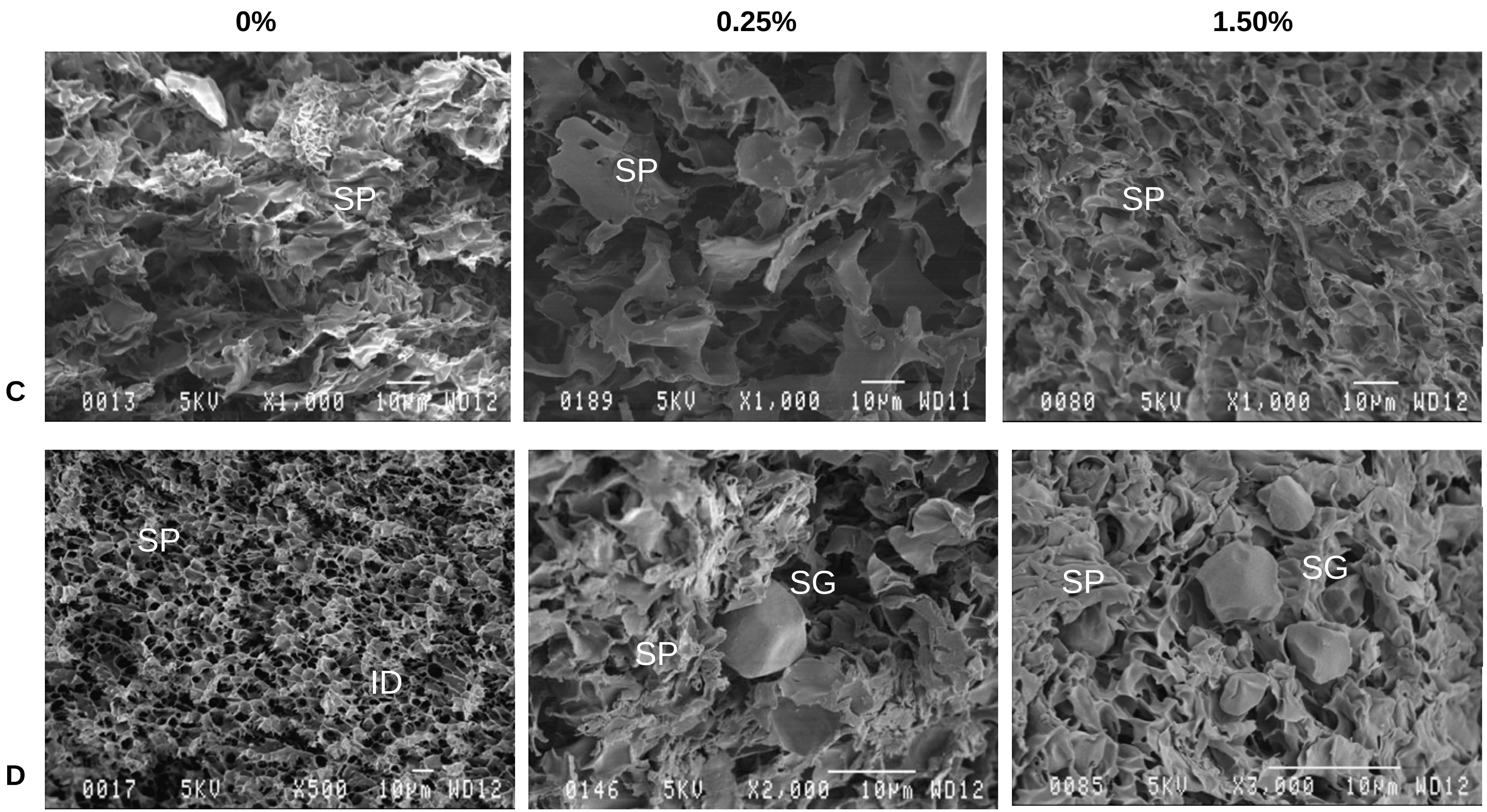


Figure 4.8 (b) (cont) Scanning electron micrographs showing the effect of stearic acid addition (0.25% and 1.50%) on maize starch sampled at C – trough and D – 2nd peak, SG, raw starch granules ; SP, starch paste; ID, Ice crystal damage

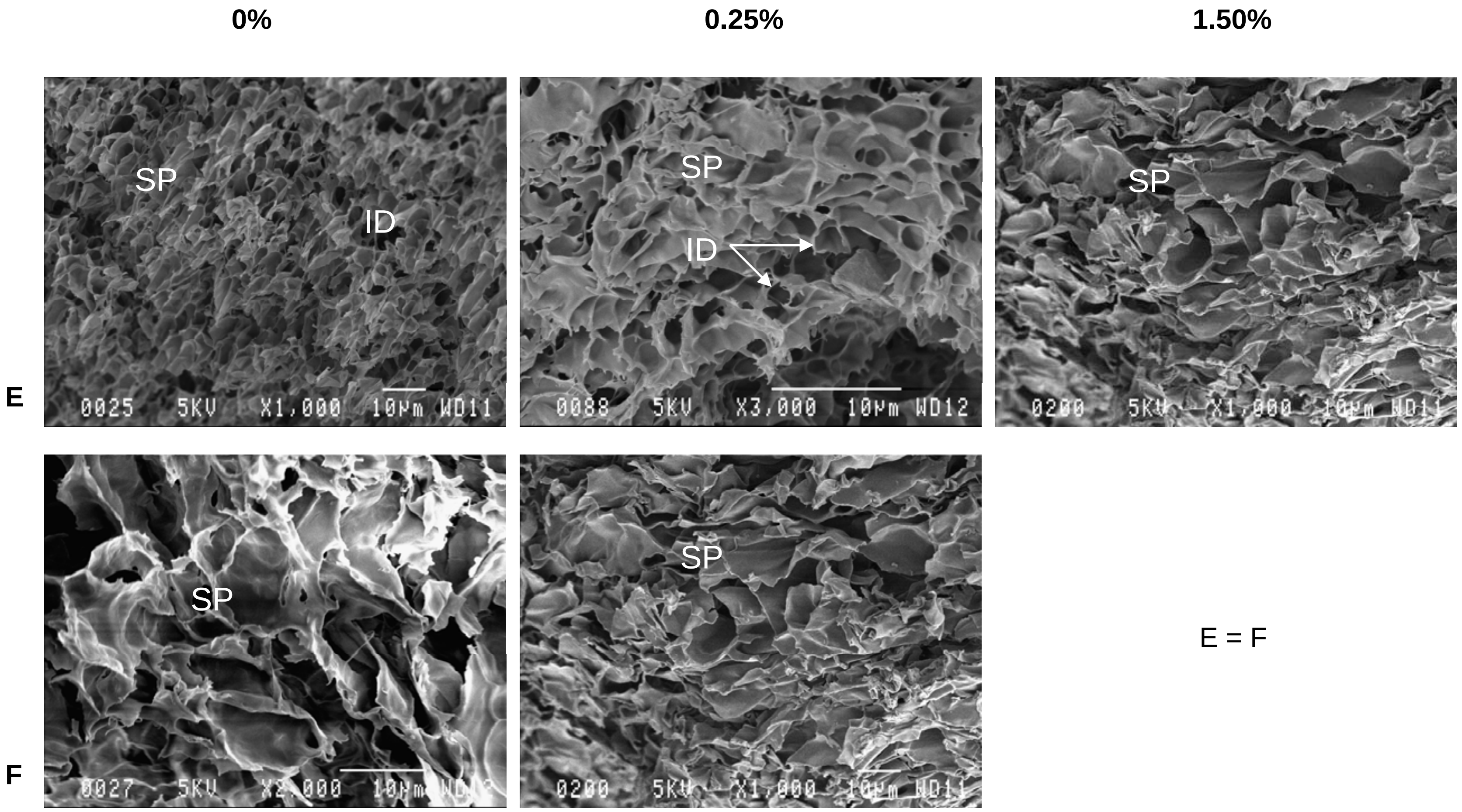


Figure 4.8 (b) (cont) Scanning electron micrographs showing the effect of stearic acid addition (0.25% and 1.50%) on maize starch sampled at E- after 2nd peak and F- end of pasting, SP, starch paste; ID, Ice crystal damage

4.3.4 DSC of tef and maize starches

For the tef starch control the endotherm representing gelatinization had a T_o (onset temperature), T_p (peak temperature) and T_c (conclusion temperature) of 60°C ,67°C and 75°C, respectively (Figure 4.9a). For the maize starch control the endotherm representing gelatinization had a T_o, T_p and T_c of 65°C, 71°C, 79°C, respectively (Figure 5.8b). Bultosa (2003) found the gelatinization temperature ranges to be 68°C-74°C-80°C for tef and 65°C-73°C-80°C for maize starch, for T_o, T_p and T_c , respectively. In the present study the starch samples were defatted, mixed with ethanol and heated to 40°C. This along with differences in grain variety could account for the slight differences in the gelatinization temperatures.

There was no significant *(p>0.05)* change in the T_p and T_c with the addition of 0.25% stearic acid to tef (Figure 4.9a) and maize starch (Figure 4.9b) starches, sampled before pasting (A). For tef and maize starches modified with 1.50% stearic acid and sampled before pasting (point A) it was difficult to determine the true T_o , T_p and T_c as the shape of the DSC thremogram representing the endothermic heat flow was different compared to the control and 0.25% stearic acid. A sharp decrease in the endothermic flow was observed from the estimated 'T_o' to reach T_p for both tef and maize starch. This unusually abrupt endothermic decrease for tef and maize starches modified with 1.50% stearic acid could possibly be as a result of the melting of stearic acid, as it melts at 69°C (Karkalas *et al*., 1995). The melting endotherm of stearic acid could therefore possibly have overlapped with the gelatinization endotherm of the starch, giving the unusual shape. For tef and maize starch (control, 0.25% and 1.50% stearic acid) sampled before pasting (point A) no other changes in heat flow were observed. For tef and maize starch controls and modified with stearic acid (0.25% and 1.50%) sampled at points C (trough), D (second peak) and F (final viscosity) no endothermic peaks for gelatinization were observed indicating that all these starch samples had gelatinized.

There was a difference in the endothermic heat flow for tef starch modified with 0.25% and 1.50% stearic acid and sampled at the trough (point C) (Figure 4.10a) compared to the control. For both 0.25% and 1.50% stearic acid, possible small changes in endothermic heat flow was observed at approximately 100°C. In contrast, stearic acid (0.25% and 1.50%) did not affect the endothermic heat flow of maize starch sampled at point C (Figure 4.10b).

For tef starch modified with 1.50% stearic acid and sampled at the second peak (point D) (Figure 4.11a) a small change in heat flow was observed at 105°C (T_p), whereas no endotherms were observed for 0.25% and control. For maize starch no endotherms were observed for control, 0.25% and 1.50%. However, the endothermic heat flow at a concentration of 1.50% stearic acid differed and seemed to decrease from approximately 100°C, whereas the heat flow for control and 0.25% seemed to remain linear to 120°C.

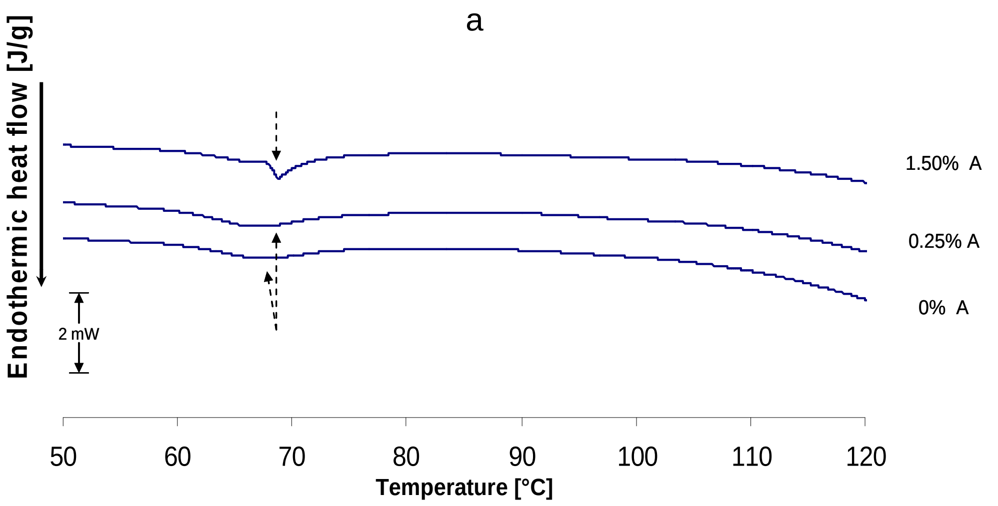
Two possible small endotherms, observed as nonlinear changes in the endothermic heat flow with peak transition temperatures at approximately 103°C and 112°C were observed for tef starch modified with 1.50% stearic acid and sampled at the end of pasting (point E) (Figure 4.12a) compared to the control and 0.25% stearic acid. Maize starch control and 0.25% sampled at the end of pasting (point E) did not seem to have any possible transitions and the heat flow remained linear to 120°C. With 1.50% stearic acid, however, a possible change in heat flow was observed at 110°C (T_p). The endotherms observed for tef and maize starch sampled at the end of pasting were, however, very small in size and difficult to identify.

Tef and maize starches modified with 1.50% stearic acid and sampled at the end of pasting (point E) was allowed to equilibrate overnight (Figure 4.13). Two separate endothermic peaks (Figure 4.13a) at approximately 99-107°C and 109-117°C were observed for tef starch. For maize starch, one endothermic peak at approximately 109-115°C was observed (Figure 4.13b).

The changes in endothermic heat flow observed for tef (at approximately 99°C-117°C) and maize (at approximately 109°C-115°C) starches modified with 1.50% stearic acid and sampled at point D (second peak) and E (end of pasting) are in range of those temperatures reported for the dissociation of type II amylose-stearic acid complexes by Elliason & Krog (1985), Raphaelides & Karkalas, (1988), Billiaderis & Galloway (1989), Karkalas *et al*. (1995) and Tufvesson *et al.* (2003). Type I amylose-stearic acid complexes dissociate at 94°C to 104°C and type II complexes dissociate at 100–125°C (Raphaelides & Karkalas, 1988; Zobel, 1988b and Karkalas *et al*., 1995).

As stated, the second viscosity peak has been attributed to the formation of amylose-lipid complexes (Nelles *et al*., 2000). Endothermic peaks observed for tef and maize starches sampled at point D (second peak) possibly indicate the formation and dissociation of type II complexes between amylose and stearic acid. Changes in endothermic heat flow observed for the starches sampled at the end of pasting could indicate that more stable amylose-stearic acid complexes were formed that did not dissociate and were present throughout the holding phase up to the end of pasting. Allowing the starch samples to equilibrate overnight could have allowed for the formation of more stable complexes, which would give more distinct DSC transitions upon reanalysis. The small size of the endotherms observed for tef and maize starches , compared to those observed by other authors, such as Karkalas *et al*. (1995) can be attributed to the difference in the concentration of stearic acid used (5%) by these authors and their preparation of pure amylose-stearic acid complexes.

No endothermic peaks corresponding to starch retrogradation at temperatures below the gelatinization temperature range (Karim *et al*., 2000) (50-70°C) were observed for tef or maize starch sampled at any point and modified with 0.25% and 1.50% stearic acid.



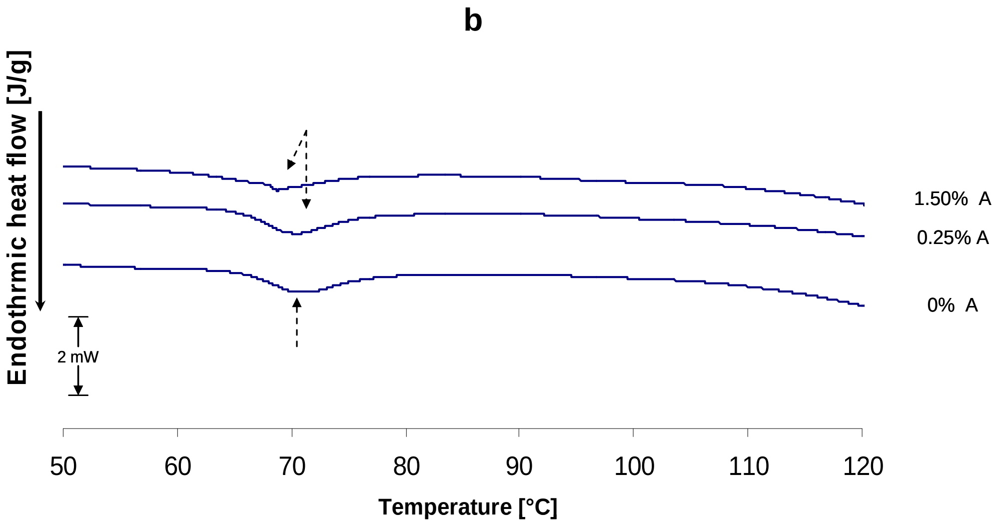


Figure 4.9 DSC thermograms showing the effect of stearic acid addition (0.25% & 1.50%) on (a) tef and (b) maize starch sampled at point A (0 min/raw starch). The dashed arrows indicate the gelatinization endotherms

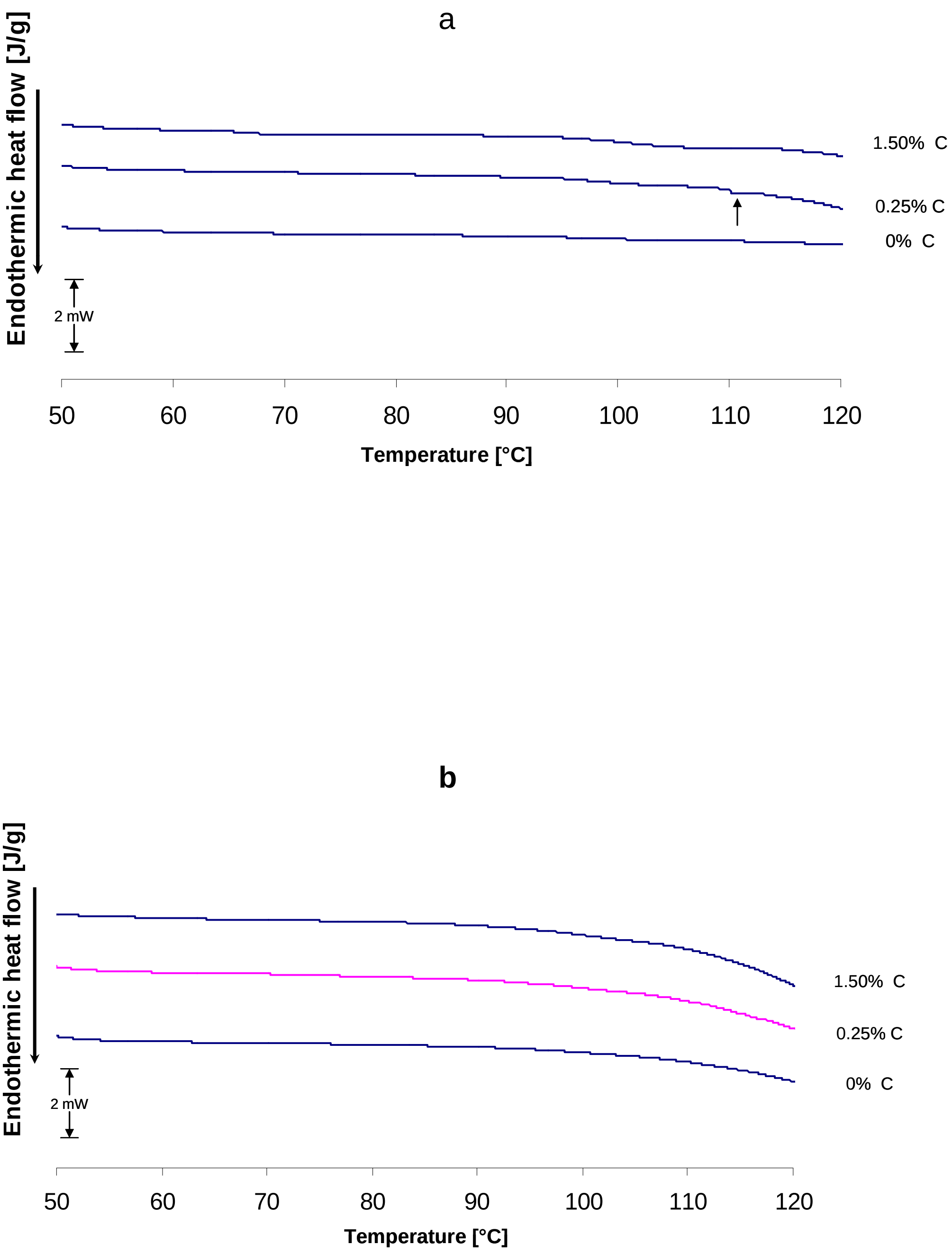
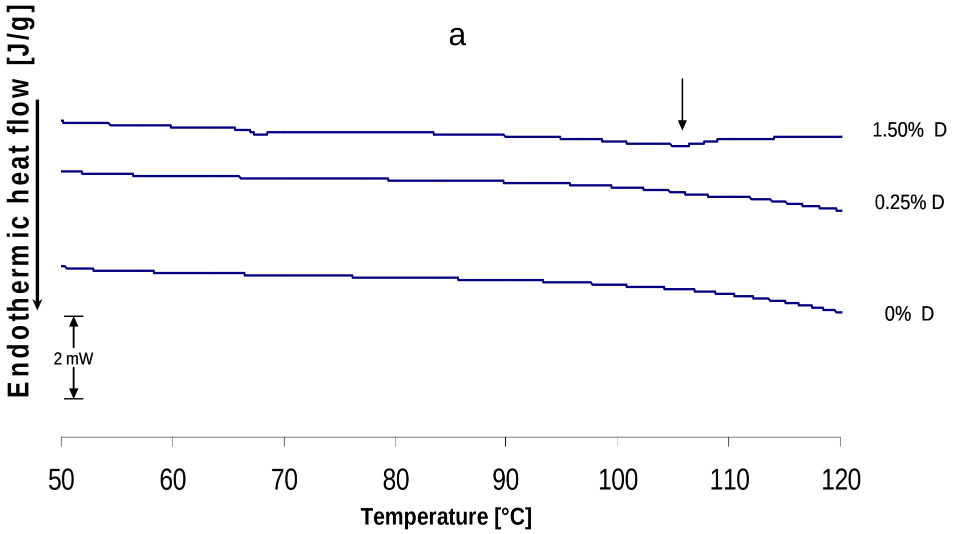


Figure 4.10 DSC thermograms showing the effect of stearic acid addition (0.25% and 1.50%) on (a) tef and (b) maize starch sampled at point C (trough) during pasting. The arrow indicates possible changes in heat flow corresponding to transitions of amylose-lipid complexes



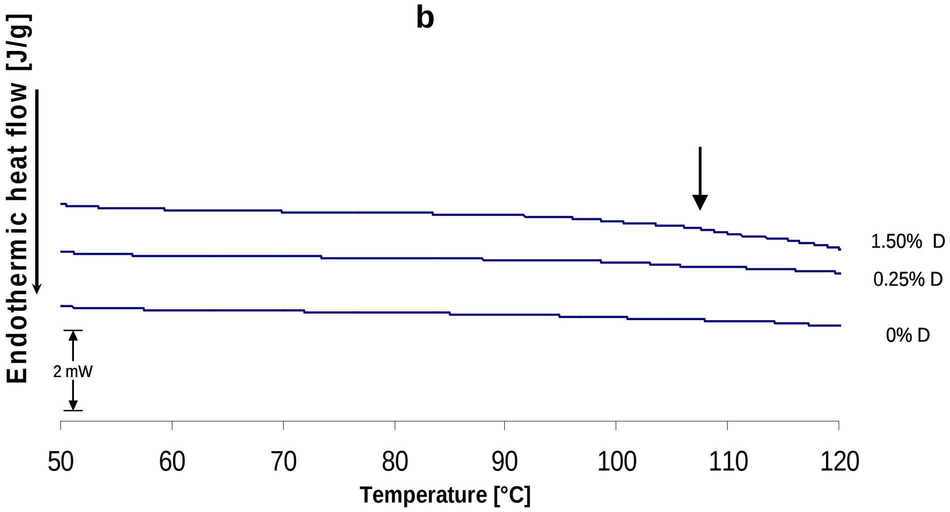
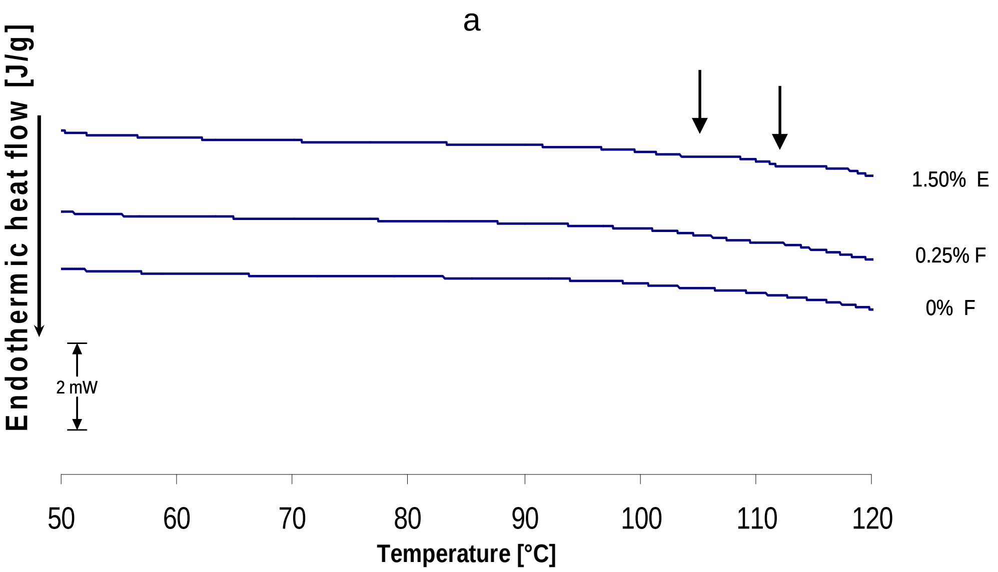


Figure 4.11 DSC thermograms showing the effect of stearic acid addition (0.25% and 1.50%) on (a) tef and (b) maize starch sampled at point D (2nd peak) during pasting. The arrow indicates possible changes in heat flow corresponding to transitions of amylose-lipid complexes



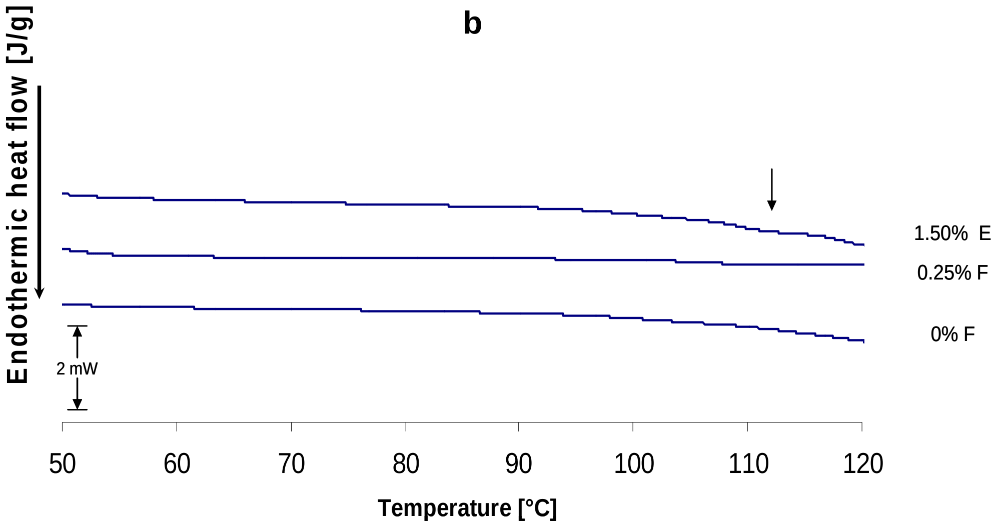


Figure 4.12 DSC thermograms showing the effect of stearic acid addition (0.25% and 1.50%) on (a) tef and (b) maize starch sampled at point E (1.50%) and F at the end of the pasting cycle. The arrows indicate possible changes in heat flow corresponding to transitions of amylose-lipid complexes.

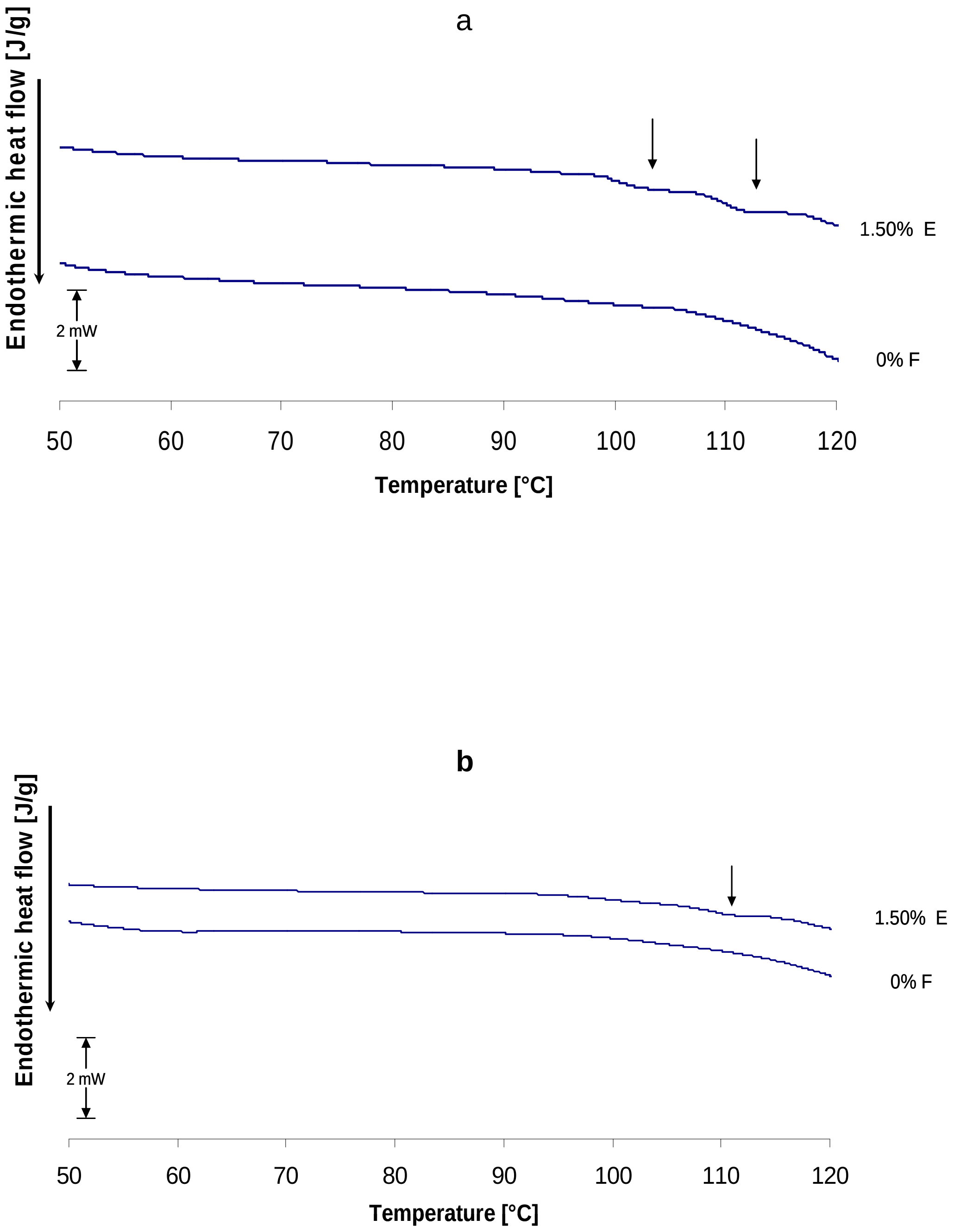


Figure 4.13 DSC thermograms showing the effect of stearic acid addition (1.50%) on (a) tef and (b) maize starch sampled at point E (1.50%) and F (at the end of the pasting cycle) and allowed to equilibrate overnight. The arrows indicate possible changes in heat flow corresponding to the transitions of amylose-lipid complexes

4.3.5 X-Ray Diffraction (XRD) of tef and maize starches

The X-ray diffractograms of tef (Figure 4.14) and maize (Figure 4.18) starch controls sampled before pasting (point A) showed peaks typical of the A type starch pattern. Zobel (1988) described the distinguishing peaks for the A type starch crystallinity to be at 0.58, 0.52 and 0.38 nm (5.8, 5.2 and 3.8 Å). Similar peaks were observed for tef (Figure 4.14) and maize (Figure 4.18) starches modified with stearic acid (0.25% and 1.50%) and sampled before pasting. These A type patterns observed matched the A type pattern observed by D'Silva (2009) for tef and maize starches modified with stearic acid and sampled before pasting. Stearic acid therefore did not seem to affect starch crystallinity prior to pasting in the RVA. For the pasted tef and maize starch samples (B, D & E/F), these A type crystallinity peaks were not observed, indicating that native starch crystalline structure was lost during pasting.

The X-ray diffractograms of tef (Figure 4.15) and maize (Figure 4.19) starch controls sampled at the first peak (point B) during pasting showed peaks at 0.44 nm and 0.68 nm (4.4 Å and 6.8 Å). These peaks (0.44 nm and 0.68 nm) were also observed for tef and maize starches modified with 0.25% and 1.50% stearic acid. The size of the peaks with 1.50% stearic acid was larger in area compared to the control and 0.25%, which were similar in size.

For tef (Figure 4.16) and maize (Figure 4.20) starch controls sampled at the second peak (point D) during pasting peaks were observed at 0.44 nm and 0.68 nm. Additionally, a peak at 1.2 nm (12 Å) was observed for maize starch at this sampling point. Similar peaks, at 0.44 nm and 0.68 nm (4.4 Å and 6.8 Å) were observed for tef and maize starches modified with 0.25% and 1.50% stearic acid. An additional 1.2 nm (12 Å) peak was also observed for maize starch modified with stearic acid (0.25% and 1.50%). A level of 1.50% stearic acid gave peaks larger in size compared to control and 0.25% stearic acid.

For the tef (Figure 4.17) and maize (Figure 4.21) starch controls and 0.25% stearic acid sampled at the end of pasting (point F) no peaks matching the V type X-ray pattern were observed. However, for the tef starch modified with 1.50% stearic acid and sampled at the end of pasting (point E) peaks were observed at 0.44 nm and 0.68 nm (4.4 Å and 6.8 Å). Similarly maize starch (1.50% stearic acid) sampled at point E gave peaks at 0.44 nm and 0.68 nm (4.4 Å and 6.8 Å), but also at 1.2 nm (12 Å).

These results agree with those reported by D'Silva (2009) who observed peaks at 0.44 nm for tef and maize starch controls and peaks at 0.68 nm and 1.2 nm for tef and maize starch modified with 0.25% stearic acid. The distinguishing peaks indicative of amylose-lipid complexes are formed at 0.44 nm, 1.2 nm and 0.68 nm (Zobel, 1988). The 4.4 Å (0.44 nm) peak is regarded as being the first indication of formation of amylose-lipid complexes. The peaks formed at 0.44, 1.2 and 0.68 nm (4.4 Å, 12 Å and 6.8 Å) for tef and maize starches modified with stearic acid (0.25% and 1.50%) possibly indicates that amylose had taken part in forming inclusion complexes with stearic acid, thereby resulting in the V_h type starch crystalline structure The characteristic amylose-lipid complex peaks formed for tef and maize starch control samples could be as a result of complexes formed between amylose and endogenous lipids.

The larger area of the diffractogram peaks observed for tef and maize starch modified with 1.50% stearic acid (compared to 0.25% and control) and sampled at points B, D and F could be due to the higher concentration of stearic acid and therefore more fatty acid available for complexation with amylose. The largest increase in peak area compared to the control and 0.25% diffractograms was observed for tef (Figure 4.16) and maize (Figure 4.20) starches sampled at the second peak (point D) during pasting. The increase in area of these peaks is further evidence that during the second peak formation, possibly more amylose-stearic acid complexes formed. The peaks for tef (Figure 4.17) and maize (Figure 4.21) starches (1.50% stearic acid) sampled at the end of pasting (point E) possibly indicate the presence of more stable Type II complexes that did not completely dissociate before the end of the pasting cycle. This further supports the DSC results (Section 4.3.4) where transition endotherms corresponding to the melting of Type II complexes where observed for tef and maize starch modified with 1.50% stearic acid and sampled at the end of pasting (point E).

The X-Ray diffractograms for tef (Figure 4.14) and maize (Figure 4.18) starches modified with 1.50% stearic acid and sampled before pasting (point A) showed peaks at approximately 0.407 nm and 0.368 nm (4.07 Å and 3.68 Å). These peaks are identified as pure uncomplexed stearic acid crystalline patterns (Figure 5.13). Similarly Zabar *et al*. (2009) also observed an additional peak at 2θ = 21.7° (0.48 nm). For tef (Figure 4.15) and maize (Figure 4.19) starches sampled at the first peak (point B) during pasting stearic acid peaks were also observed, but these were smaller compared to those observed at point A. The stearic acid peaks were however not observed for tef and maize sampled at the second peak (point D) and at the end of pasting (point E). The reason the stearic acid peaks formed at points A and B could be due to an excess of stearic acid that possibly selfassociated to form aggregates and therefore did not take part in complex formation during the first few minutes of pasting at 50°C. Tang and Copeland (2007) studied the effect of stearic acid on wheat starch and observed stearic acid aggregate X-ray diffraction peaks at 21.5° and 23.9°. They suggested that above certain concentrations lipids tend to self associate. The absence of these stearic acid peaks for starch samples at the second peak (point D) at the end of pasting (point E), could possibly indicate that during the holding phase at elevated temperatures (91°C) most of the stearic acid had taken part in complex formation with amylose, therefore increasing the amount of complexes formed, consequently also increasing the size of the diffractogram peaks corresponding to V_h peaks. This could be the reason for the larger areas of the V_h peaks, as more stearic acid would be available to complex with amylose.

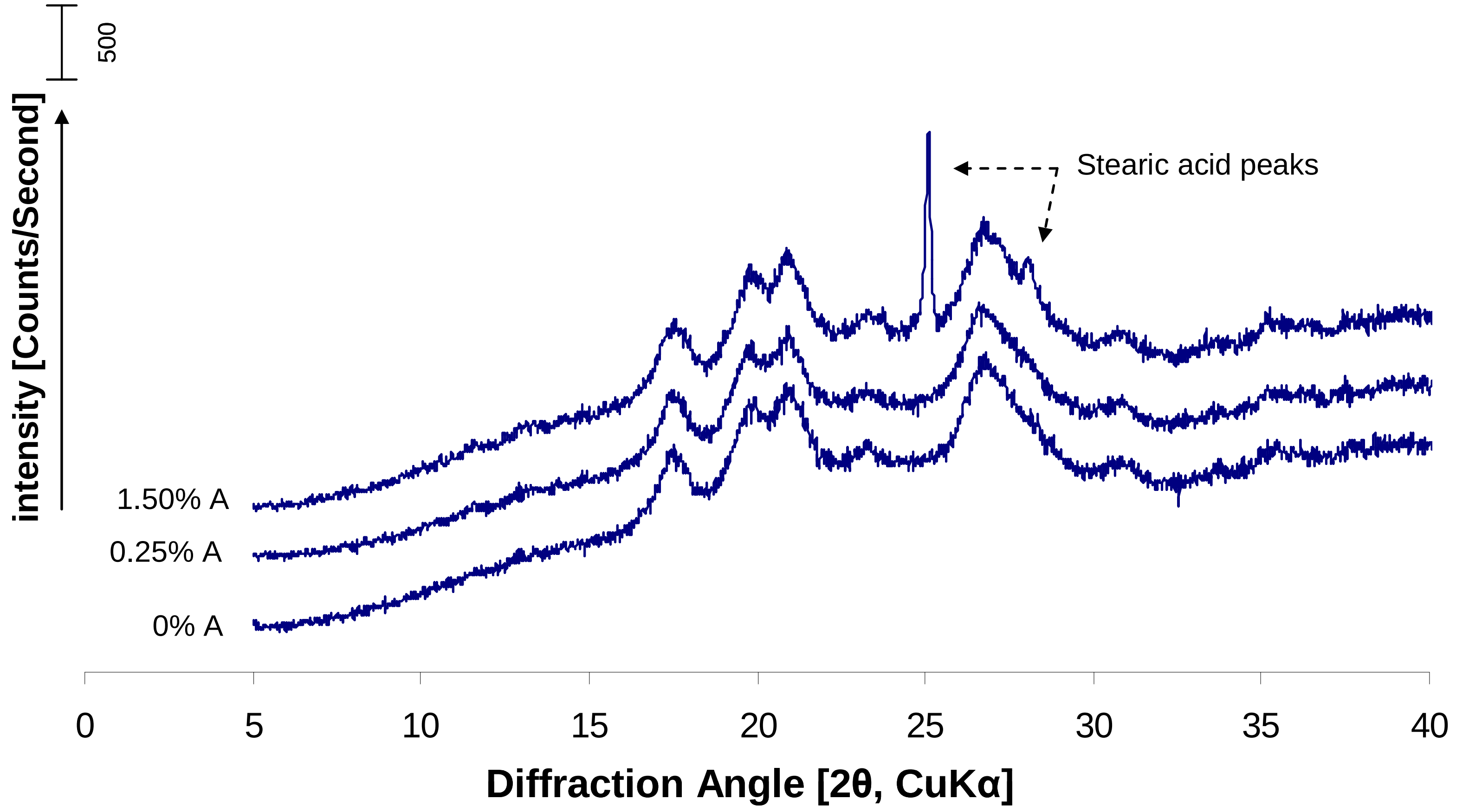


Figure 4.14 X-ray diffractogram showing the effect of stearic acid addition (0.25% and 1.50%) on tef starch sampled at point A (0 min/ raw starch); dashed arrows indicate peaks characteristic of stearic acid

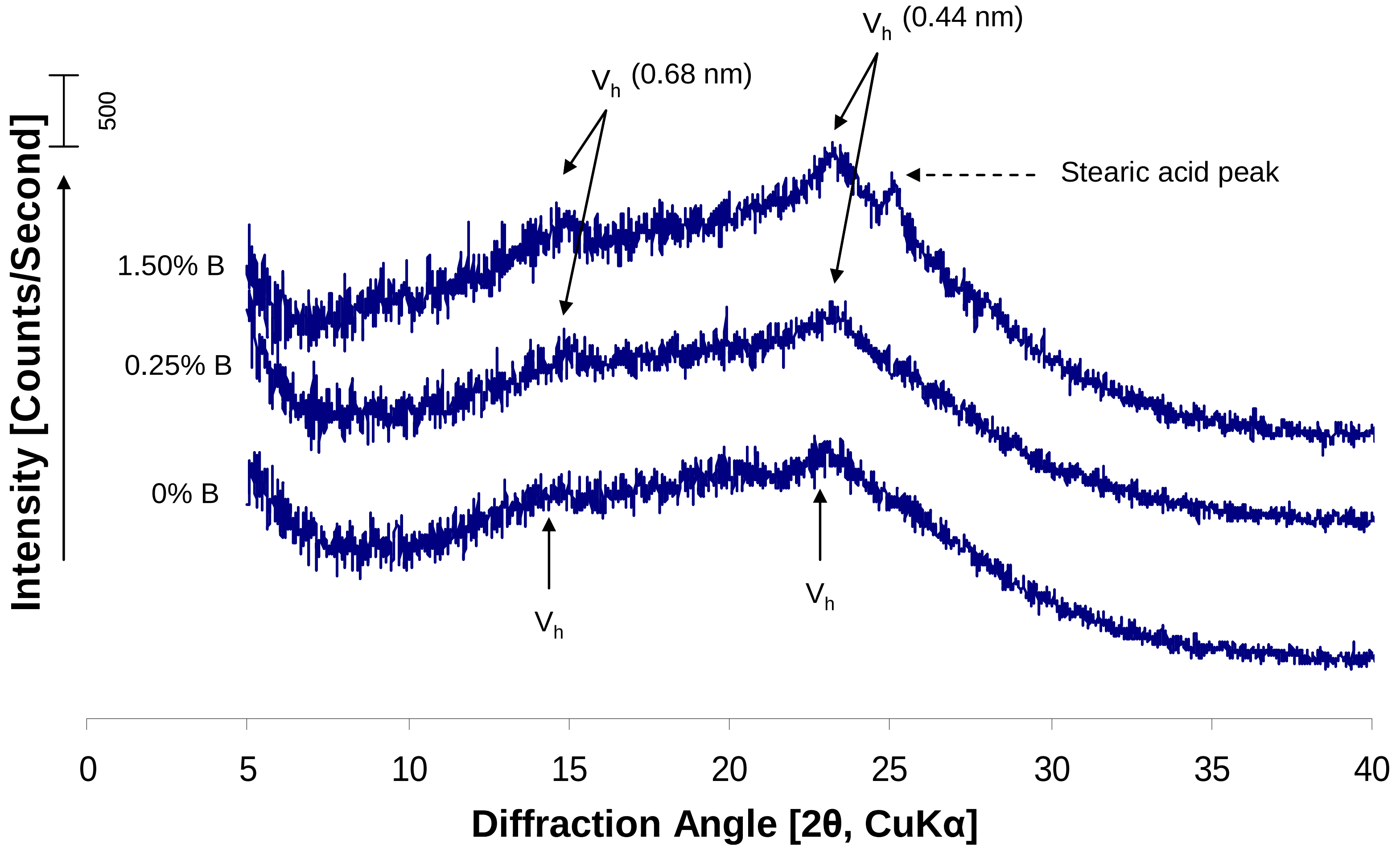


Figure 4.15 X-ray diffractograms showing the effect of stearic acid addition (0.25% and 1.50%) on tef starch sampled at point B (first peak) during pasting. Solid arrows indicate peaks characteristic of Vh amylose, dashed arrows indicate peaks characteristic of stearic acid.

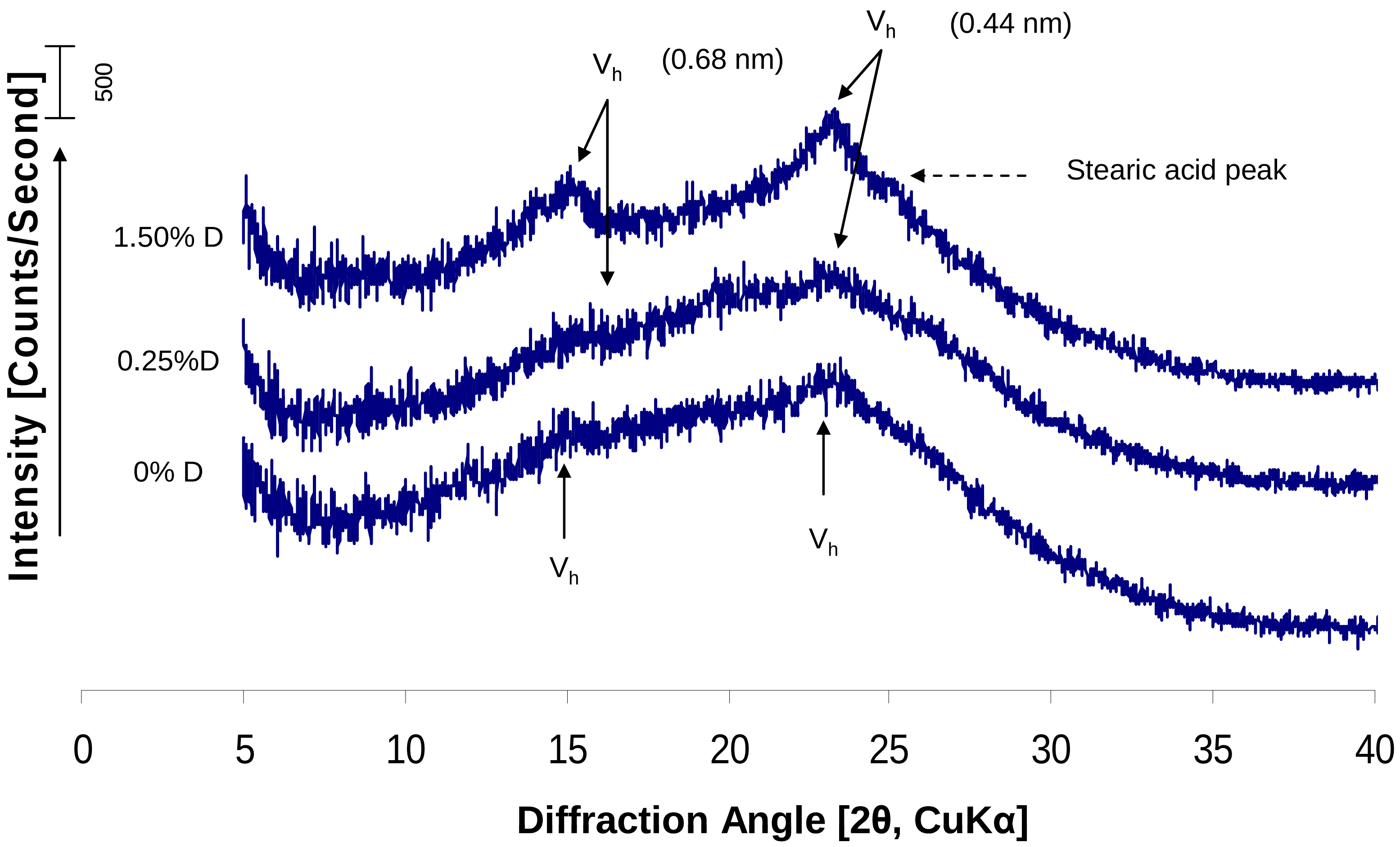


Figure 4.16 X-ray diffractograms showing the effect of stearic acid addition (0.25% and 1.50%) on tef starch sampled at point D (second peak) during pasting. Solid arrows indicate peaks characteristic of Vh amylose, dashed arrows indicate peaks characteristic of stearic acid

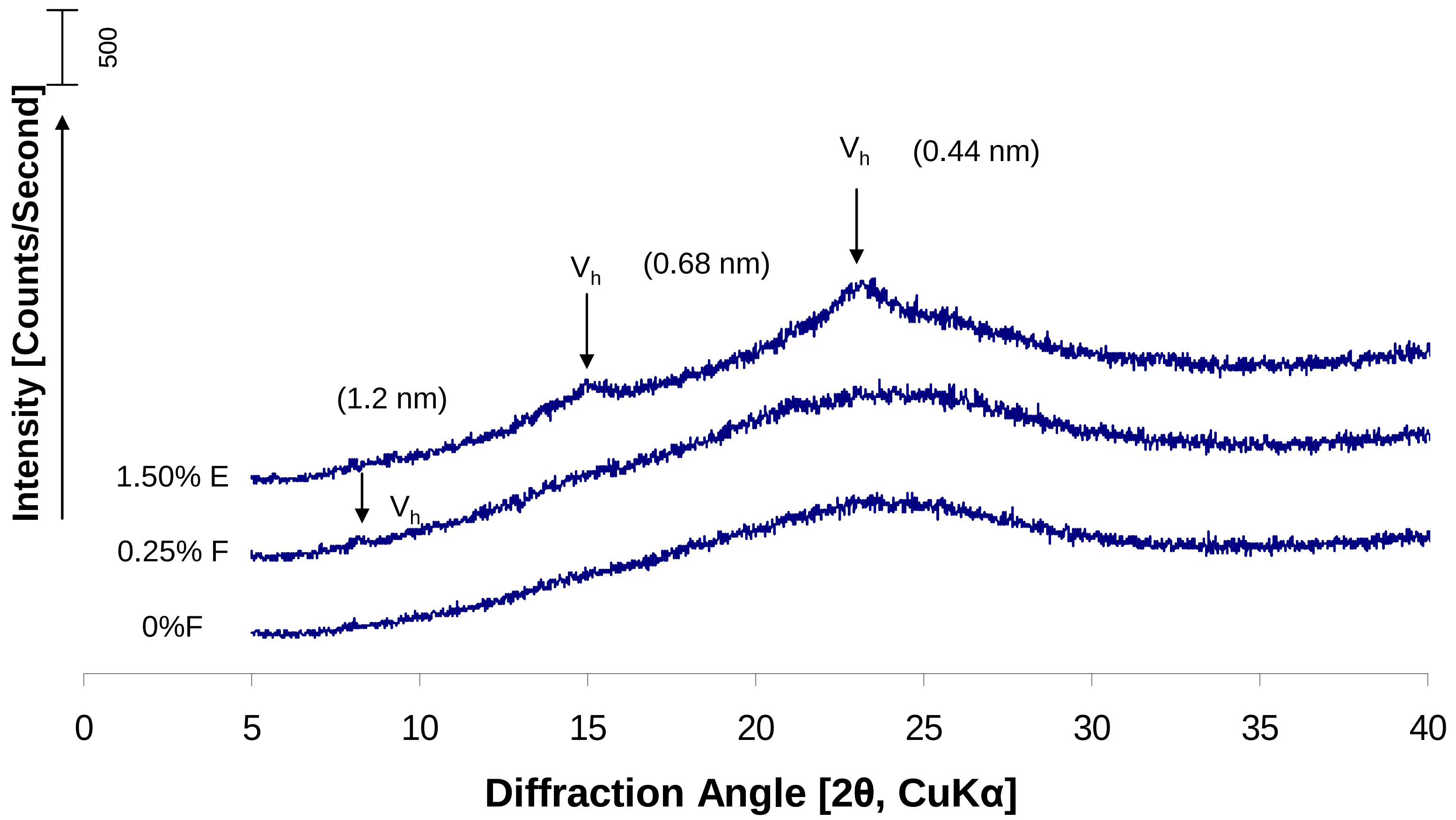


Figure 4.17 X-ray diffractograms showing the effect of stearic acid addition (0.25% and 1.50%) on tef starch sampled at E / F (end of pasting). Solid arrows indicate peaks characteristic of Vh amylose

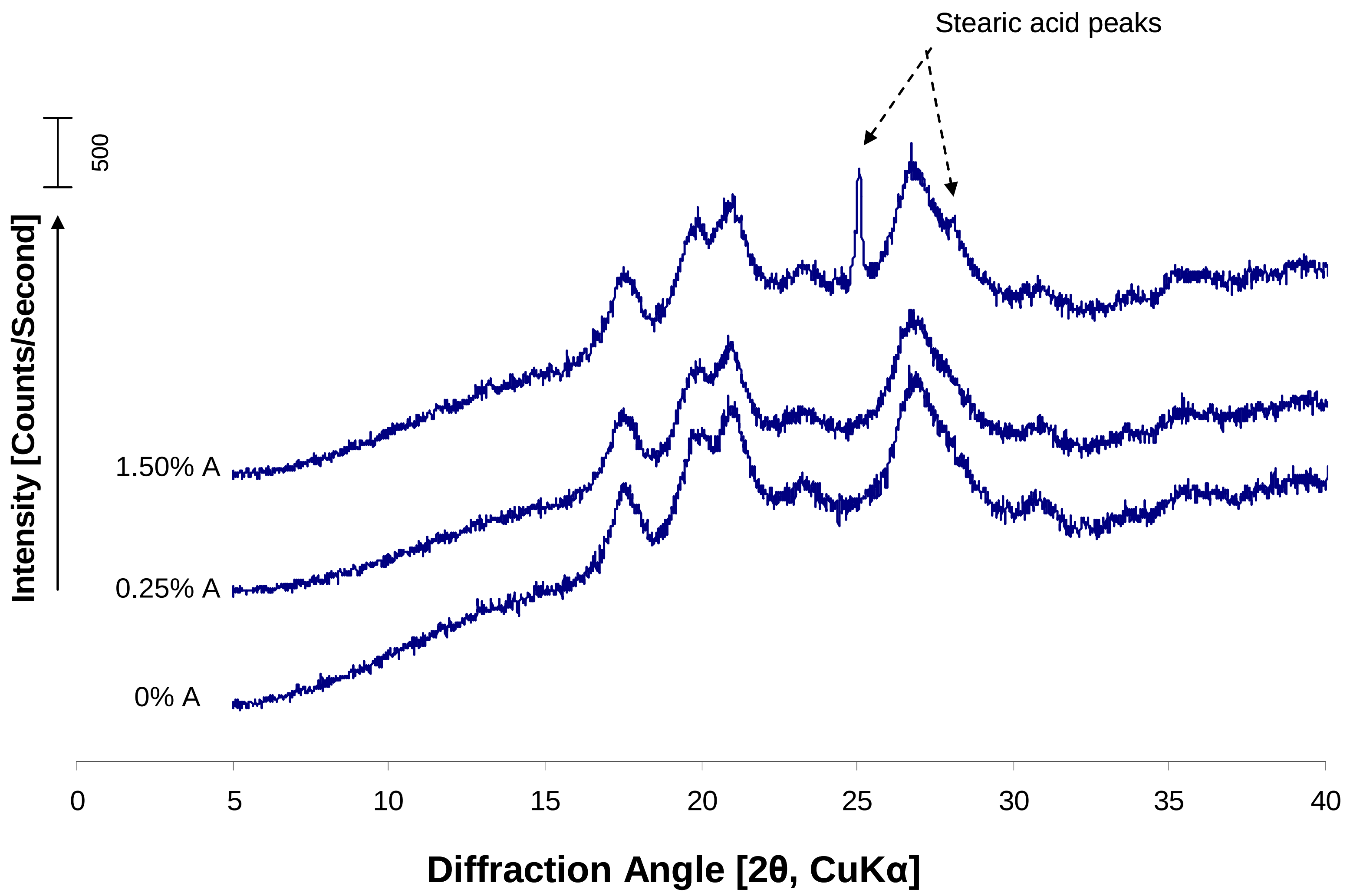


Figure 4.18 X-ray diffractograms showing the effect of stearic acid addition (0.25% and 1.50%) on maize starch sampled at point A (raw starch before pasting) ;dashed arrows indicate peaks characteristic of stearic acid

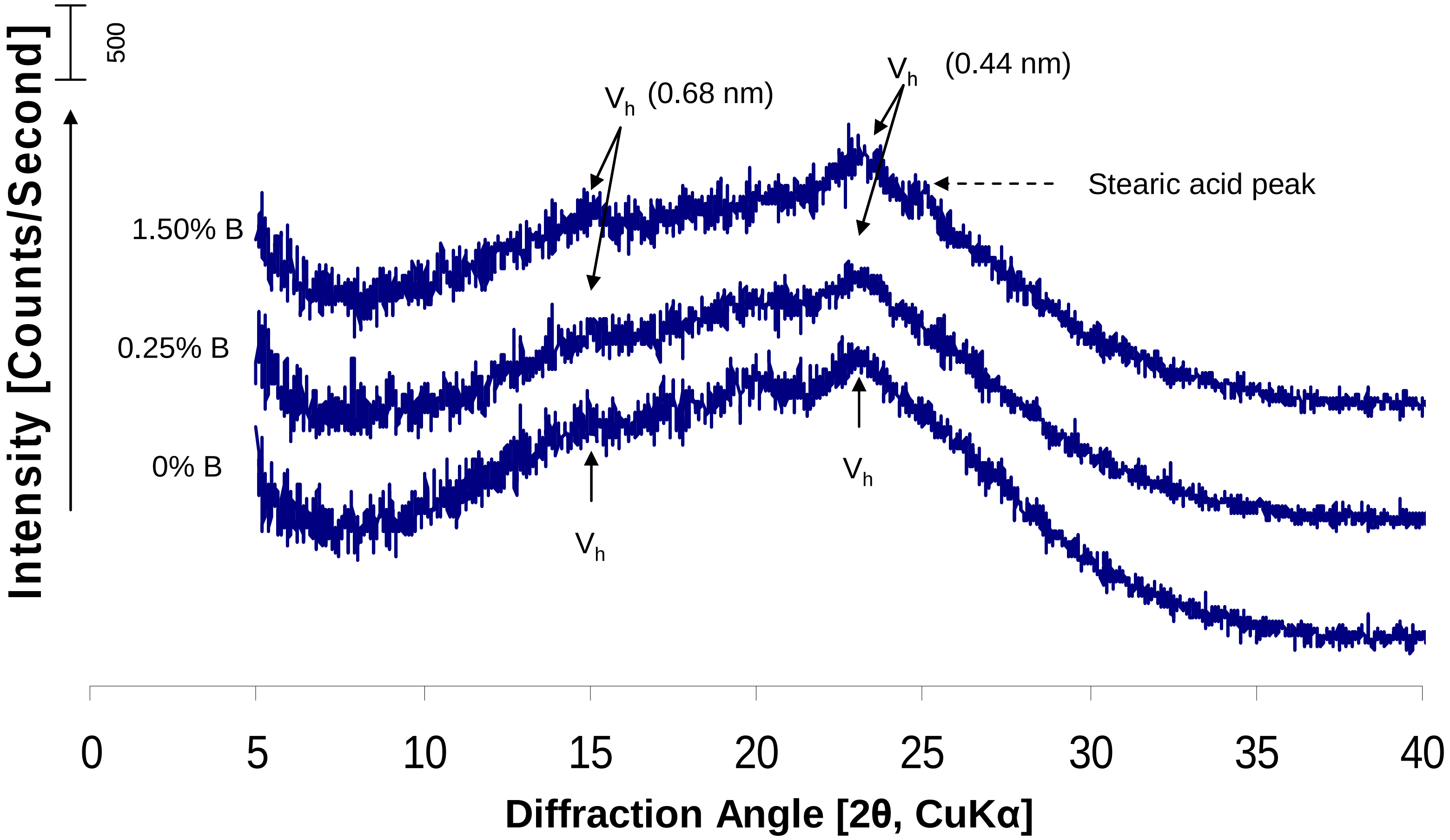


Figure 4.19 X-ray diffractogram showing the effect of stearic acid addition (0.25% and 1.50%) on maize starch sampled at point B (first peak) during pasting. Solid arrows indicate peaks characteristic of Vh amylose, dashed arrows indicate peaks characteristic of stearic acid

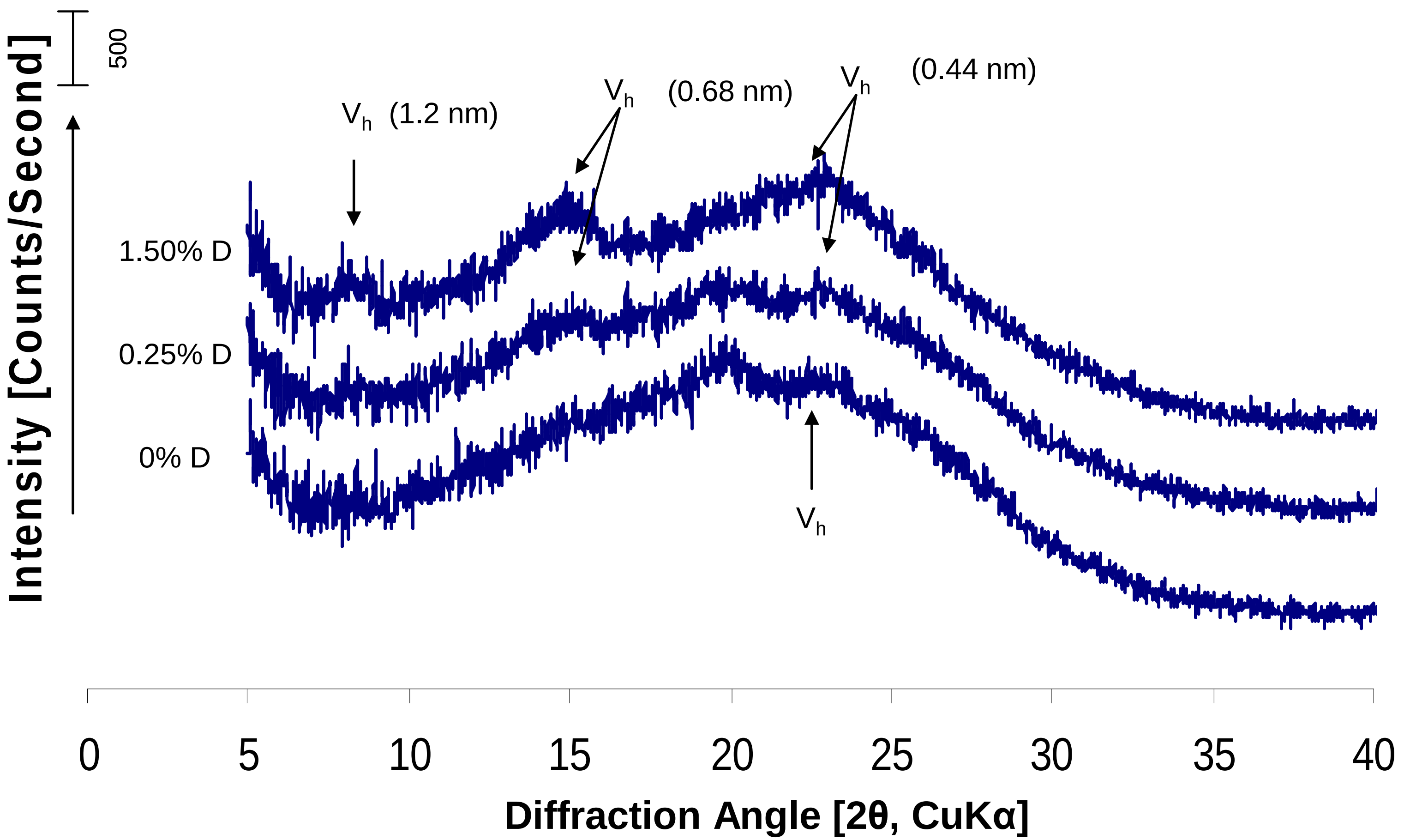


Figure 4.20 X-ray diffractograms showing the effect of stearic acid addition (0.25% and 1.50%) on maize starch sampled at point D (second peak) during pasting. Solid arrows indicate peaks characteristic of Vh amylose

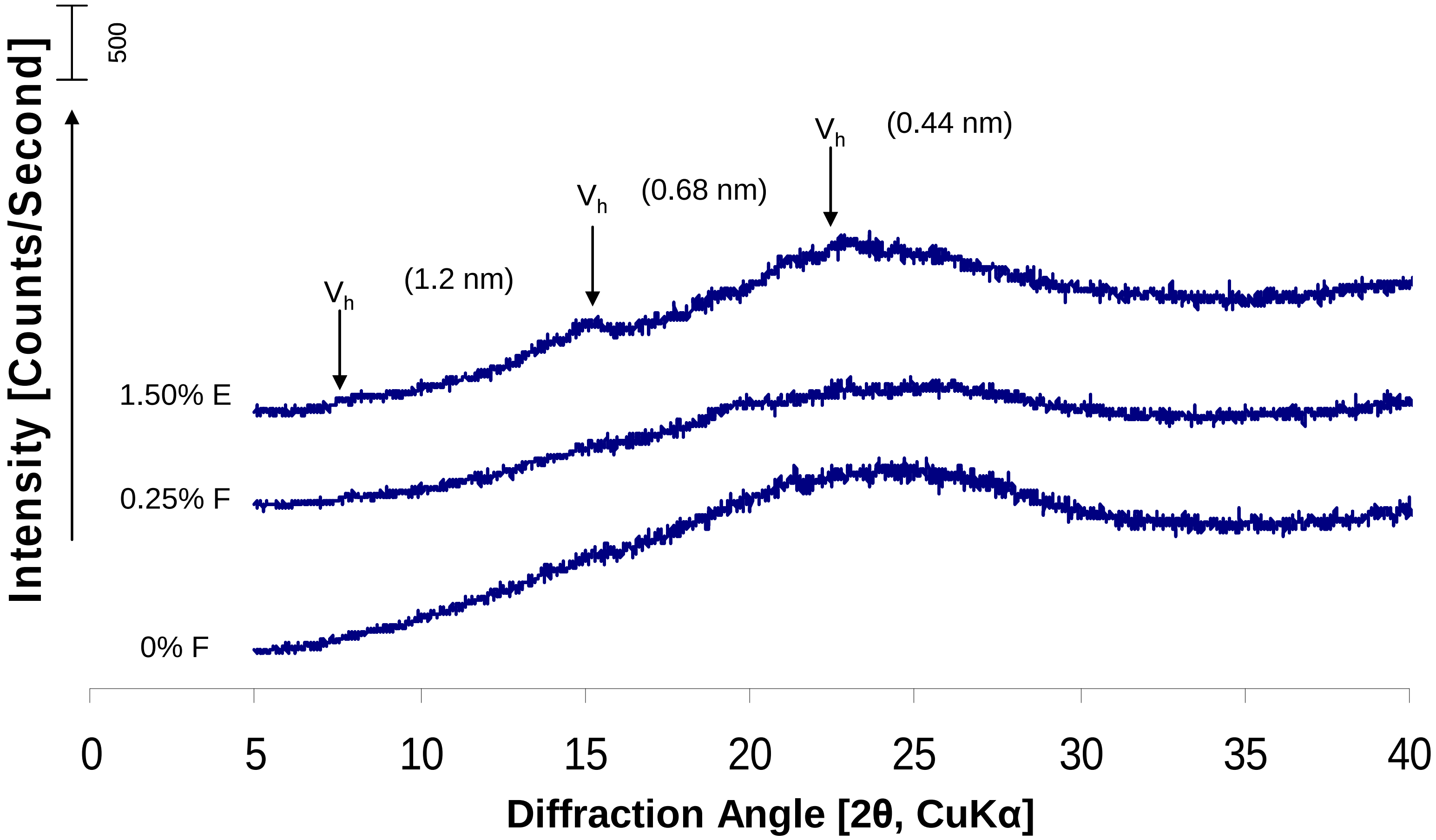


Figure 4.21 X-ray diffractograms showing the effect of stearic acid addition (0.25% and 1.50%) on maize starch sampled at point E / F (end of pasting). Solid arrows indicate peaks characteristic of Vh amylose

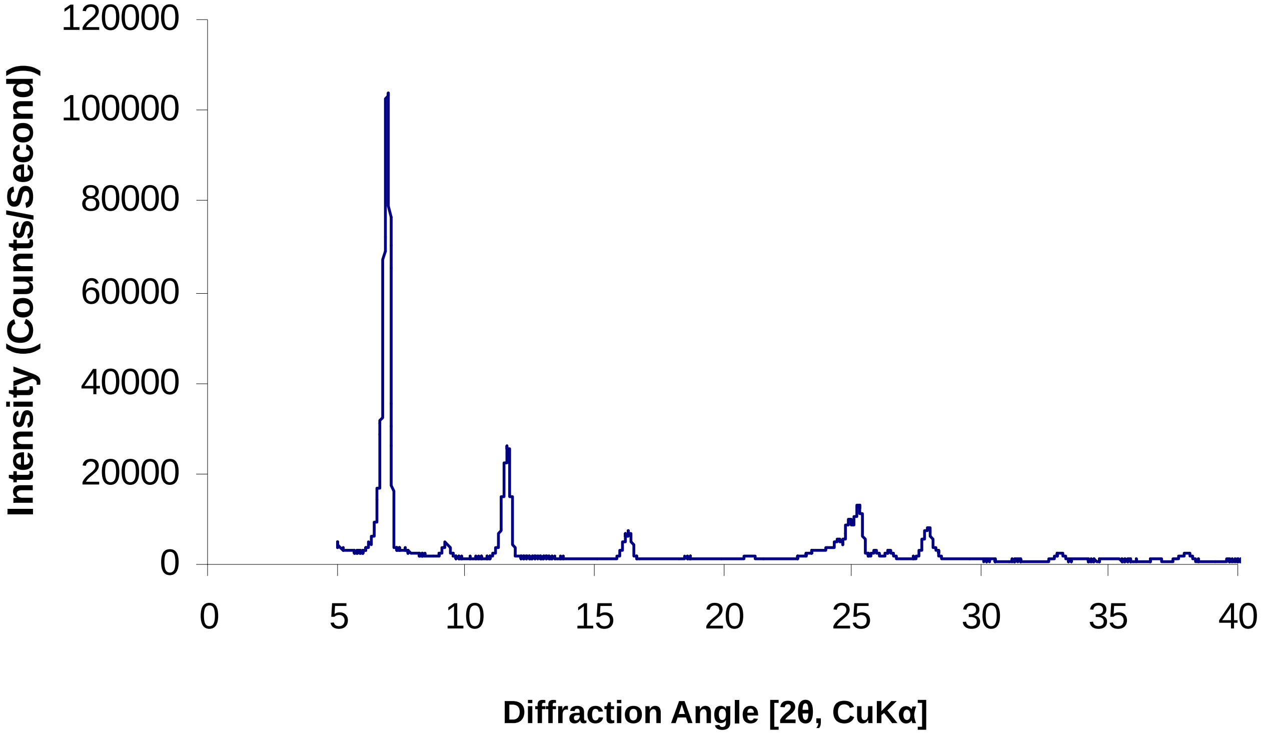


Figure 4.22 X-ray diffractogram of stearic acid

4.3.6 Conclusions

The modification of tef and maize starches with stearic acid (0.25% and 1.50%) results in the formation of a second viscosity peak during an extended holding period (2 or 4 hours at 91°C) during pasting in the RVA. The development of the second viscosity peak can be attributed to the formation of amylose-stearic acid complexes. This is because X-ray diffractograms of tef and maize starches sampled at the second viscosity peak show the characteristic V-type crystallinity peaks (at 0.44 nm, 1.2 nm and 0.68 nm). DSC data also supports the formation of amylose-stearic acid complexes. DSC thermograms with endothermic heat flow changes are observed at temperatures in range with the transition temperatures of amylose-lipid complexes (at 99-120°C). Stearic acid changes the microstructure of tef and maize starches during pasting. SEM and light microscopy show both tef and maize starches modified with stearic acid (0.25% and 1.50%) to have larger proportions of intact granules (compared to the control). Microscopy, however, does not provide evidence as to the presence of amylose-stearic acid complexes.

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CHAPTER 5
GENERAL DISCUSSION

This chapter critically reviews the major results and methodology used in the research project.

5.1 Review of methodology

The major methods used in the research project are critically reviewed below with respect to shortcomings, possible improvements and methodologies that should be considered in future. The impacts of the methodology on the results are also discussed.

Maize starch is extracted on a commercial scale with the aid of chemicals such as sodium metabisulphite and/or sodium hydroxide (Kent & Evers, 1994). Sulphur dioxide (SO_2) liberated from sodium metabisulphite (dissolved in the water wherein the maize grains are steeped) facilitates the disruption of the disulfide bonds of the protein matrix during steeping. This is believed to break down the protein matrix for easy separation of protein and starch (Kent & Evers 1994). During steeping only distilled water was used to extract tef starch. The difference in the extraction of tef compared to maize starch may have resulted in the higher protein content of tef starch compared to maize starch. The difference in protein contents of tef compared to maize starch probably did not significantly affect the results. This is because both tef and maize starches had high total starch contents, 84% and 89% respectively, compared to the small amount of protein.

The method used for the incorporation of stearic acid into tef and maize starch, has previously used by Bajner (2002), Zhou *et al*. (2007) and D'Silva (2009). This method involves adding the starches (tef and maize) to the stearic acid-ethanol mixture and placing it in a shaking water bath at 50°C for 30 min followed by evaporating off the ethanol. It is not known whether stearic acid/fatty acid diffuses into the starch granules or not. This is thought to be influenced by the presence or absence of pores on the surface of the starch granule (D'Silva, 2009). It has been reported that maize starch granules contain surface pores (Fannon *et al*., 1992), leading to channels extending into the granules (Gallant *et al*., 1997) (Figure 4.4). These pores and channels could have facilitated the diffusion of stearic acid into maize starch granules. No pores were found on the surface of tef starch granules (Bultosa, 2002) (Figure 4.4). This would therefore not allow for easy access of the stearic acid. D'Silva (2009) observed using confocal laser scanning microscopy (CLSM) that stearic acid diffused into maize starch granules (with surface pores) but not into tef starch granules (without surface pores). If the absence of pores on tef starch granules possibly affects the incorporation of stearic acid into the granules, then this may alter the effect of the stearic acid treatment. Absence of surface pores for tef may also possibly account for tef starch behaving differently from maize starch during extended pasting. This is, however, not known and therefore further research and possible further development is required into the method for incorporating stearic acid into tef starch granules. This will possibly give more information regarding the difference in the effect of stearic acid on tef starch functional properties compared to maize starch.

The RVA is widely used in determining the pasting behaviour of starch-water dispersions (Batey, 2007). The instrument measures the changes in the starch suspension viscosity, while stirring and heat is applied (Thomas & Atwell, 1999). In order to avoid clumping and settling of the starch in the edges of the canister, water was first measured into the aluminium canister, before the tef and maize starch was added. The starch suspension was then manually stirred using the paddle, prior to being placed in the RVA. This method of mixing was a precaution in order to limit clumping and settling to a minimum as this would result in high variability among repetitions, but it is difficult to completely eliminate this problem. This can negatively affect the accuracy of the pasting curves and therefore only repeatable measurements were taken as reliable data. The RVA viscosity measurements were, however, very repeatable with very few outliers (on average one in approximately every 10 to 12 measurements).

Tef and maize starch samples were heated in the RVA to 91°C and held at this temperature for 2 hours or 4 hours, depending on the concentration of stearic acid used. The reason for heating to 91°C and not to 95°C was to prevent boiling of the samples, as water boils in Pretoria at approximately 95°C because of the high altitude (approximately 1400 metre).

Loss of moisture would have occurred throughout the long holding period as pasting in the RVA is not a closed system. If a considerable amount of moisture was lost the starch sample viscosity would have been an overestimate. However, loss of moisture for the stearic acid modified tef and maize starches was minimal (approximately 1 g was lost from the canister during the extended pasting profile). Additionally, the loss in moisture would be relative to the control (unmodified) tef and maize starches, as these would also lose moisture throughout pasting in the RVA. Therefore the viscosity measurements would still be valid.

The tef and maize starch samples taken at various stages during pasting (in the RVA) were immediately frozen in liquid nitrogen (-180°C). Cryogens, such as liquid nitrogen are used to structurally and chemically preserve the samples (Brooker, 1990). Immediate freezing should provide starch samples that were representative of the starch structure during that specific stage of pasting. The starch samples were also stored in liquid nitrogen to further preserve them for light microscopy. This practical method of freezing and storage should prevent the starch samples from undergoing possible structural changes over time, which could possibly negatively affect the results, such as retrogradation.

No evidence of retrogradation for tef and maize starches frozen and preserved using liquid nitrogen was observed with DSC analysis. While the use of liquid nitrogen for freezing and storage of starch samples was relatively practical, SEM did show evident ice-crystal damage to the starch paste networks (Figure 4.8a and d). This could possibly have been avoided by more rapid freezing of the starch pastes, thereby limiting the formation of large ice crystals. Using propane (liquid form) for this purpose could possibly minimize ice crystal formation as it has a much higher freezing rate (Bald, 1984). Additionally, ice crystal damage is not a major drawback and should not significantly have affected the results.

Light microscopy and SEM were used to determine the structural changes of tef and maize starch pastes as affected by the addition of stearic acid (0.25% and 1.50%) and also to determine the possible presence of amylose-stearic acid crystals. Nelles *et al.* (2003) used light microscopy and SEM to investigate the micro structural changes of maize starch pastes. They reported results that were in agreement with the results for this study. Fanta *et al.* (2002) and Peterson *et al.* (2006) reported micrometer sized crystals observed using light microscopy and SEM, suggested to be amylose-lipid complexes. In the current work, very few structures, that closely resembled those of Fanta *et al.* (2002) and Peterson *et al.* (2006) were observed by light microscopy for maize starch and none were observed using SEM. No such structures were observed for tef starch. However, both X-ray diffraction and DSC data suggest the presence of amylose-lipid complexes in tef and maize starch pastes. The structures observed for maize starch could possibly have been amylose-stearic acid complexes. However, due the small number observed and the absence thereof for tef starch, these could possibly also have been ungelatinized maize starch granules as their size and shape were similar to that of maize starch granules.

D'Silva (2009) did not observe any amylose-lipid structures using CLSM. It is important to note, however, that in the investigations of Fanta *et al*. (2002) and Peterson *et al.* (2006) the slow cooling (22 hours at room temperature) of jet cooked starch-lipid mixtures was essential to allow crystal growth and isolation. As stated, in the current study, starch pastes were rapidly cooled using liquid nitrogen. This could have possibly hindered the formation of larger crystalline complexes. Additionally, the amylose-lipid complexes, suggested to be responsible for the second pasting peak formation, could be nano-sized instead of micro-sized. Nano-sized structures, believed to be amylose-*n*-butanol complexes have been observed by Kim and Lim (2009). They prepared the nano-sized particles by a gravimetric filtration of a DMSO (dimethyl sulphoxide)-amylomaize starch mixture into an *n*-butanol solution over a period of 6 days at 70°C, followed by amylolysis using α-amylase. The hydrolysis using α-amylase was necessary to hydrolyze all the uncomplexed starch, leaving only the precipitate of complexed amylose. Godet *et al*. (1995) and Zabar *et al*. (2009) used a water/DMSO mixture to isolate amylose complexes. Kim and Lim (2009) used TEM to view the nano structures. Therefore, the size resolution of the light microscope and the SEM used in this study, were probably not high enough to determine the presence of any possibly nano-sized amylose-stearic acid complexes. In future, to possibly isolate amylose-stearic acid complexes (be it micro or nano-sized) from tef and maize starches, hydrolysis using α-amylase or DMSO, should be used. Atomic Force Microscopy (AFM) which has higher resolution (Baldwin *et al*., 1998) might also prove more successful in identifying the amylose-stearic acid complexes.

Differential scanning calorimetry (DSC) measures the difference in heat flow as a function of temperature between a sample and a reference, which is recorded as a peak on the DSC thermogram. In the present work, DSC was used to study the possible presence of amylose-lipid complexes formed during extended pasting of tef and maize starches modified with stearic acid (0.25% and 1.50%). Amylose-lipid complexes have been observed on the DSC thermogram as endothermic transitions above the gelatinisation temperature range (Kugimiya *et al*. 1980). In the present study, tef and maize starch samples (10 mg), conditioned to a moisture content of approximately 8%, were weighed into aluminium pans and 30 mg of water was added. Endothermic heat flow changes corresponding to melting of amylose-lipid complexes (at approximately 100-120°C) were observed for tef and maize starches (1.50% stearic acid) sampled at and after the second pasting peak (Figures 4.11 to 4.13). These possible endothermic changes were, however, small and difficult to clearly identify. Even after the DSC samples were allowed to equilibrate overnight, the possible transitions observed for both tef and maize starches, were much smaller and less defined than for those previously reported (Eliason & Krog, 1985; Raphaelides & Karkalas, 1988; Billiaderis & Galloway 1989; Karkalas *et al*., 1995 and Tufvesson *et al.,* 2003). The small changes in heat flow observed could possibly be due to only small amounts of amylose-stearic acid complexes formed. Additionally, allowing for even longer equilibration might have possibly produced larger endotherms.

The differences in the magnitude of the endothermic heat flow changes observed in the present study compared to previous reports, could be due to differences in sample preparation techniques e.g. method of addition of water, heating rate and equilibration time (Yu & Christie, 2001; reviewed by Bultosa, 2003). Water content and heating times have been found to affect the melting transitions of amylose-lipid complexes (Biliaderis *et al*., 1985, Karkalas *et al*., 1995 and Tufvesson et al., 2003). Increasing the starch-water ratio used for analysis of complexes has been found to result in larger endothermic transitions at higher temperatures (Kugimiya *et al*., 1980). In future, use of higher amounts of water for DSC analysis of tef and maize starch, may possibly result in more well defined endothermic transitions and therefore better identification of the possible amylose-stearic acid complexes. It is also important to keep in mind the limitations of DSC analysis in identifying amylose-lipid complexes. A limitation of conventional DSC analysis is its insensitivity to phase transitions below its detection limit (Lui *et al*., 2009). Therefore conventional DSC thermal analysis is unable to identify small amounts of amorphous content because of the small energy changes associated with the melting of amorphous material (reviewed by Saunders *et al*., 2004). High speed DSC (Hyper DSC) operates at heating rates of up to 500°C/min and is reported to be more sensitive (Saunders *et al*., 2004). This advanced method of thermal DSC analysis could prove useful in future investigations to better identify the presence of not only V_h – amylose but also the presence of V_a - amylose, present in tef and maize starches.

X-ray diffraction (XRD) was performed to determine if any crystalline amylose-stearic acid complexes had formed, using conditioned (as for DSC) tef and maize starch samples modified with stearic acid and ground to a fine, homogenous powder. The diffractograms obtained (Figures 4.14 to 4.19) gave peaks at 0.68 nm, 1.2 nm and 0.44 nm, characteristic of crystalline amylose-lipid complexes (Zobel, 1988b), thereby providing evidence that for both tef and maize starches (controls, 0.25% and 1.50% stearic acid) the crystalline structures may have changed from A-type to V-type (Zobel, 1988). Diffractogram peaks characteristic of amylose-lipid complexes (4.4 Å, 12 Å and 6.8 Å) were also observed by D'Silva (2009) for tef and maize starch controls and modified with stearic acid (0.25%). In the present study, it also appeared that the addition of stearic acid (1.50%) to both tef and maize starches, notably increased the size of the characteristic amylose-lipid complex peaks (0.68 nm and 0.44 nm), indicating that a higher level of stearic acid addition, possibly resulted in larger proportions of crystalline amylose-stearic acid complexes formed.

For the purpose of this study, XRD proved mostly successful in determining the possible presence of crystalline amylose-stearic acid (V_h) complexes. XRD is, however, not able to provide information regarding the presence of type I (amorphous) complexes (Eliasson & Wahlgren, 2004). In future studies wide angle X-ray scattering (WAXS) and small angle X-ray scattering (SAXS) could be used to providing additional information of the size and the structure of the amylose-stearic acid complexes. WAXS is used to study crystal structure (Derycke *et al*., 2005), SAXS (using x-rays or neutrons) is useful because of its ability to explore larger lengths scales (nanometre distances and upwards) (Donald *et al*., 2001). SAXS studies the properties of amylose-lipid complexes at a nano-scale, which enables the investigation of less ordered systems (reviewed by Donald *et al*., 2001; Zabar *et al*., 2009). For example, SAXS reveals that there is a regular constant repeat, in a wide range of starch granules, which appears at 9 nm (Eliasson & Wahlgren, 2004).

5.2 The effect of addition of stearic acid on the microstructure and thermal properties of tef and maize starch pastes

The overall effects of stearic acid (0.25% and 1.50%) on tef and maize starch pasting properties and microstructure of pastes sampled at various points during pasting are summarised in Table 5.1.

The second viscosity peak observed for both tef and maize starches (modified with stearic acid) and its increase in size with increased level of stearic acid indicates the possible role that stearic acid plays in its formation, possibly in the form of amylose-stearic acid complexes. The XRD and DSC results further shows possible evidence of amylose-lipid complexes present in tef and maize starches modified with stearic acid. While the light microscopy and SEM data did not show evidence of amylose-stearic acid complexes, their presence in the form of nano-sized structures, cannot be disregarded.

The above mentioned reasons show that both starch and stearic acid play an important role in the formation of the second peak and also indicates the presence of amylose-lipid complexes, but it is still not evident whether the second peak is due to the formation of amylose-stearic acid complexes.

Table 5.1 Overall major effects of stearic acid (0.25% and 1.50%) addition on the pasting properties and microstructure of tef and maize starch sampled at various points during pasting

Effect of stearic acid addition on:	**Tef**	**Maize**
Pasting properties	Formation of a 2nd viscosity peak during extended holding period. Peak size increased from 0.25% to 1.50%	Formation of a 2nd viscosity peak during extended holding period Peak size increased from 0.25% to 1.50%
Microscopical appearance	Increased proportions of intact swollen granule up to second peak formation. No crystalline structure observed	Increased proportions of intact swollen granules up to second peak formation Some birefringent structures observed for 1.50% paste sampled at second peak
X-Ray pattern	Indicated formation of V-type crystallinity for pastes sampled at the first peak, 2nd peak and end of pasting.	Indicated formation of V-type crystallinity for pastes sampled at the first peak, 2nd peak and end of pasting.
DSC thernogram	Differences in endothermic heat flow observed (compared to control) at temperatures in range with the melting of amylose-lipid complexes.	Differences in endothermic heat flow observed (compared to control) at temperatures in range with the melting of amylose-lipid complexes.

Figure 5.1 is a diagrammatic representation of the possible role that amylose-lipid complexes and possible other factors play in the second peak formation. It is worth mentioning that the starch-stearic acid suspension subjected to the RVA pasting profile did not only contain amylose and stearic acid, but was a more complicated system. This system also contained amylopectin that could also play roles individually or in combination with the amylose-lipid complexes, in the second peak formation. For example, the large viscosity increase at a level of 1.50% stearic acid could be as a result of excess stearic acid dispersed in the system, leading to an increased viscosity. There is also the possibility that the second increase in viscosity was also in part a result of breakdown of starch granules and hydrolysis of the amylose molecules, possibly as a result of extended exposure to high temperatures during the holding period. The microscopy data from this study, similar to the reports of Nelles *et al*. (2003), support this assumption, as the starch granules seemed intact up to second peak formation. Additionally it is important to note that not only amylose, but also amylopectin has the ability to form inclusion complexes with lipids (Eliasson & Wahlgren, 2004).

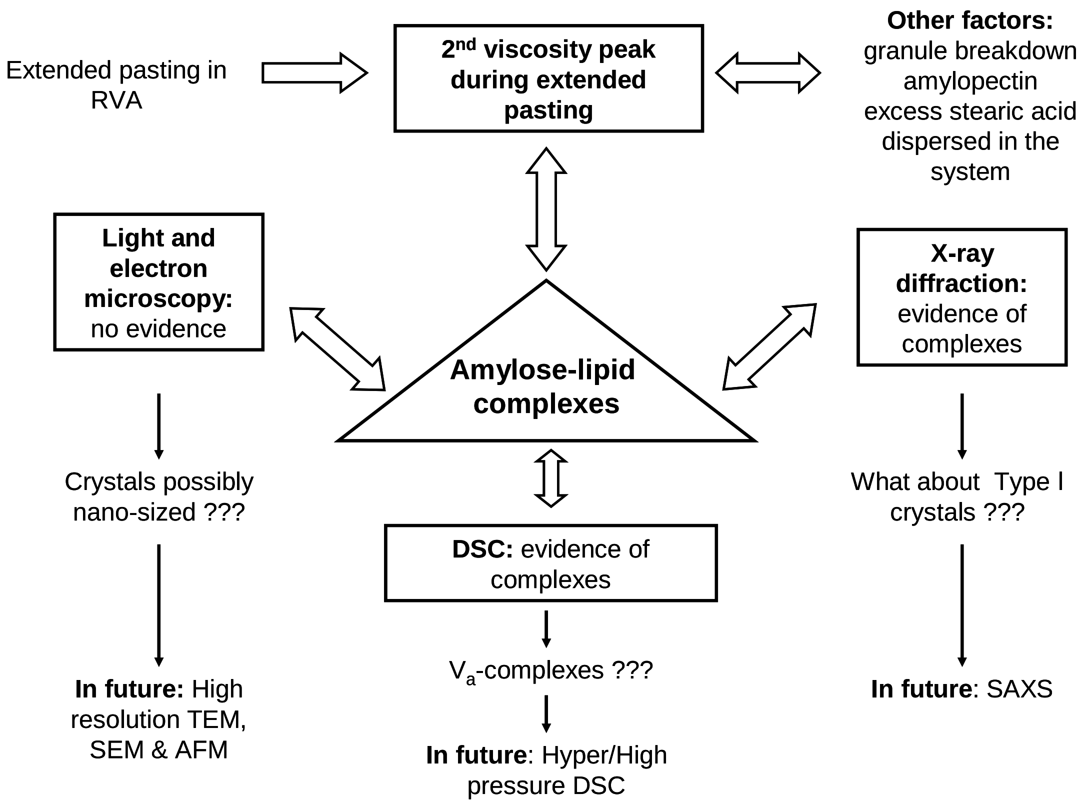

Figure 5.1 Diagram summarising possible factors influencing the 2nd peak formation, the role of amylose-lipid complexes, methods used for its detection and the possible future methods that could be applied to further investigate the role of amylose-stearic acid complexes in the formation of the second viscosity peak for tef and maize starches

5.3 Possible application of stearic acid modified tef and maize starches as fat replacers

Fat replacers/mimetics are substances that replace or improve one or more functional properties of fat in reduced fat foods but without supplying the added calorific value (Muir *et al*., 1999). The increased paste viscosity and significantly reduced retrogradation (especially with 1.50%) are properties of tef and maize starches (modified with stearic acid) that make them potentially useful as fat replacers/mimetics (as described below).

Small sized starch granules may potentially be used as fat replacers (Lucca & Tepper, 1994). This makes tef starch granules (1–10 µm) possibly suitable to mimic the texture of homogenised fat globules and act as a fat replacer (Bultosa, 2003). Additionally, amylose-lipid complexes could be used in reduced fat products as fat mimetics. This is because the size and shape of the crystalline amylose-lipid complexes could possibly enable them to physically mimic lipid micelles giving a similar texture and mouthfeel (Dang & Copeland, 2003), while the lipid fraction may also act as carriers of flavour (Tari *et al*., 2003 ; Singh & Kim, 2009).

Retrogradation is the major drawback of using starch as a fat replacer (Gudmundsson, 1992; Lucca & Tepper, 1994). Retrogradation is the Increase in starch viscosity during cooling (setback) due to starch molecules realigning and re-establishing ordered structure (Thomas & Atwell, 1999). Syneresis occurs as these molecules regain ordered structure and squeeze out water and is responsible for staling of bread and baked foods. The modification of tef and maize starches with 1.50% stearic acid resulted in the paste viscosity continuously decreasing during the cooling period (Figures 4.2 and 4.3). The final viscosity was therefore very low. Thus it appears that stearic acid hindered the amylose molecules from realigning. Retrogradation, due to crystallisation of amylopectin, (during later stages of storage) will, however, still occur and it is important to mention that although the formation of amylose-lipid complexes may reduce retrogradation, its direct addition of pre-prepared amylose-lipid complexes to starch containing products will not prevent it (reviewed by Eliasson & Wahlgren, 2004). Singh and Byars (2008) observed reduced syneresis of yoghurt produced with starch-lipid composites. Reduced retrogradation of stearic acid modified starches would possibly make the suitable fat replacers in low viscosity products such as salad dressings and drinking yoghurts.

The modification of tef and maize starches with stearic acid (0.25% and 1.50%) resulted in a large increase in viscosity. Singh and Kim (2009) reported that incorporating starch-lipid composites (emulsions formed from jet cooked starch and fat) in the production of yoghurt resulted in an increase in viscosity and creamier texture. This means that, with the addition of only a small amount of stearic acid (0.25%), less starch is needed to greatly increase the viscosity of a paste, then when only native starch is used. Therefore, the contribution of calories from the starch source is also reduced, without compromising on the desired product texture. Additionally, amylose-lipid complexes have reduced susceptibility to enzyme digestion (Seneviratne & Biliaderis, 1991). Therefore, stearic acid modified starches that are less digestible by the human digestive enzymes do not contribute the total calorific value. Hence, these modified starches could also find application in low fat foods as a bulking agent.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Amylose-stearic acid complexes has a role to play in the production of the second peak viscosity when tef and maize starches modified with stearic acid (0.25% and 1.50%) are pasted for an extended holding period of 2 or 4 hours at 91 °C in the RVA. This second viscosity peak increased in size with increasing levels of stearic acid, suggesting more complexes formed at higher stearic acid concentration. In addition, XRD and DSC analysis show evidence of the formation of amylose-lipid complexes at the second peak viscosity.

Stearic acid seems to change the microstructure of tef and maize starches during pasting. SEM and light microscopy indicate that stearic acid seems to reduce granule breakdown of tef and maize starches. SEM and light microscopy, however, does not seem to provide evidence of micron sized crystalline amylose-lipid complexes, possibly because the complexes are nano scale. Thus, the use of microscopic techniques with higher resolution such as Atomic Force Microscopy) and SAXS may prove more successful in identifying amylose-lipid complexes, as these may be nano sized.

Tef starch modified with stearic acid can be potentially useful as a fat replacer/mimic. This is because of the increased paste viscosity, small sized, granules as well as the possible formation of micro/nano sized amylose-stearic acid complexes.

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