3.3 Cowpeas cooking characteristics as affected by micronisation temperature: a study of the physicochemical and functional properties of starch[3]

Abstract

Moisture-conditioned (41 %) Bechuana white cowpeas were micronised to three temperatures (130, 153 and 170 °C). Cooking properties of the cowpea seeds and the role of starch-related properties were studied. Micronisation (41 % moisture, 130, 153 and 170 °C) in all cases significantly reduced the cooking time and thus the time required for the cowpea seeds to attain a suitably soft texture. This was attributed in part to the significant improvement in rate of water absorption during cooking and starch pregelatinisation as evidenced by loss of birefringence and increased susceptibility of the cowpeas starch to α-amylase digestion. However, micronisation of moisture-conditioned (41 % moisture) cowpeas to 170 °C resulted in a severe reduction in pasting properties of the cowpea flour possibly due to starch depolymerisation and/or amylose associated crosslinking. Due to these changes, M-170 °C cowpea seeds required a longer cooking time than the other two moisture-conditioned and micronised samples. Hence flour prepared from cowpeas treated at M-170 °C had less starch functionality.

Key words: cowpea, starch, micronisation temperature, gelatinisation, amylose,

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3.3.1 Introduction

Cowpeas are rich in starch and protein that provide relatively affordable protein and energy in carbohydrate-based diets of most developing countries (Giami, 2005). Cowpeas are consumed in different forms, one of which is in boiled stews (Taiwo, 1998), which is common in southern Africa. Dried cowpeas prepared in such a way require extended cooking times to attain a palatable texture and flavour that is acceptable to consumers. The cooking time (CT_{50}) of dried cowpeas ranges from 31 to 160 min (Akinyele *et al*., 1986), which translate into increased fuel energy use and time required. In most urban and peri-urban areas of Africa, fuel wood is the main source of energy and is in short supply.

Micronisation is one of the possible processes that could be used as a precooking treatment for cowpeas to decrease cooking time. According to Zarkadas and Wiseman (2001), micronisation refers to a short-time, high-temperature infrared processing method that utilises moisture and temperature to achieve conditions for optimum cooking and starch gelatinisation. As reported in section 3.2, micronisation of Bechuana white cowpeas (41 % moisture) to 153 °C reduced the cooking time by 44 %. Similar reductions in cooking time of moisture-conditioned and micronised legumes have been reported in Laird lentils (50 %) (Arntfield *et al*., 2001) and split peas (30 %) (Cenkowksi & Sosulski, 1998).

The effectiveness of micronisation of moisture conditioned legumes as a precooking treatment for legumes is dependent on several processing parameters. The final micronisation temperature is one of the critical process parameters during hydrothermal processing of leguminous seeds. It has been shown that after 15 min of cooking, lentils (33 % moisture) micronised to a high temperature (170 °C) coupled with lower final moisture content (7 %) had a significantly harder texture than lentils micronised (33 % moisture) to 138 °C (Arntfield *et al*., 2001) leading to increased cooking time to attain acceptable softness. This phenomenon impacts negatively on the effectiveness of the process and needs to be further investigated. Since starch is one of the major macromolecular constituents of cowpeas (Kerr *et al*., 2001), this research studied the effect of high (170 °C), medium (153 °C) and low (130 °C) micronisation temperatures on physicochemical and functional properties of cowpea

starch with the aim of explaining the possible differences in cooking characteristics of moisture-conditioned cowpeas micronised to higher temperatures.

3.3.2 Materials and methods

3.3.2.1 Raw materials

Bechuana white (cream colour) cowpeas (11.4 g per 100 seeds, 6mm by 5mm) supplied by Agricol (Potchefstroom, South Africa) were cleaned to remove chaff, shrivelled and broken seeds. The cleaned seeds were packed in propylene bags and stored at 4 °C until the time of use.

3.3.2.2 The hydrothermal process

The cowpeas were micronised according to the method described in section 3.2.2.2. Cowpea seeds were moisture conditioned to 41 % moisture (wb) by steeping in deionised water (1:5 w/v) for 6 h and holding for 12 h at 22 °C for the moisture to equilibrate throughout the seeds. The moisture-conditioned cowpeas were micronised in 160 g batches for 3, 6 and 8 min to final surface temperatures of 130 (M-130 °C), 153 (M-153 °C) and 170 °C (M-170 °C), respectively, using a tabletop microniser (Technilamp Pty, Johannesburg, South Africa). Following micronisation, the micronised cowpeas had 25 %, 12 % and 5.0 % moisture content for the M-130 °C, M-153 °C and M-170°C treatments, respectively. Figure 3.3.1 shows the experimental flow diagram. Cooking properties of the unmicronised and micronised (M-130 °C, M-153 °C and M-170 °C) samples were determined immediately. Since the cowpeas micronised to 130 °C had retained higher moisture content, these samples were freeze dried in preparation for milling.

3.3.2.3 Determination of moisture content

Moisture content of raw, tempered and micronised cowpea seeds was determined according to the method of Ajibola *et al*. (2003) as reported in section 3.1.2.1.

3.3.2.4 Determination of water absorption during soaking

Water absorption during soaking was determined according to a modified method of Agbo *et al*. (1987) as reported in section 3.1.2.4.

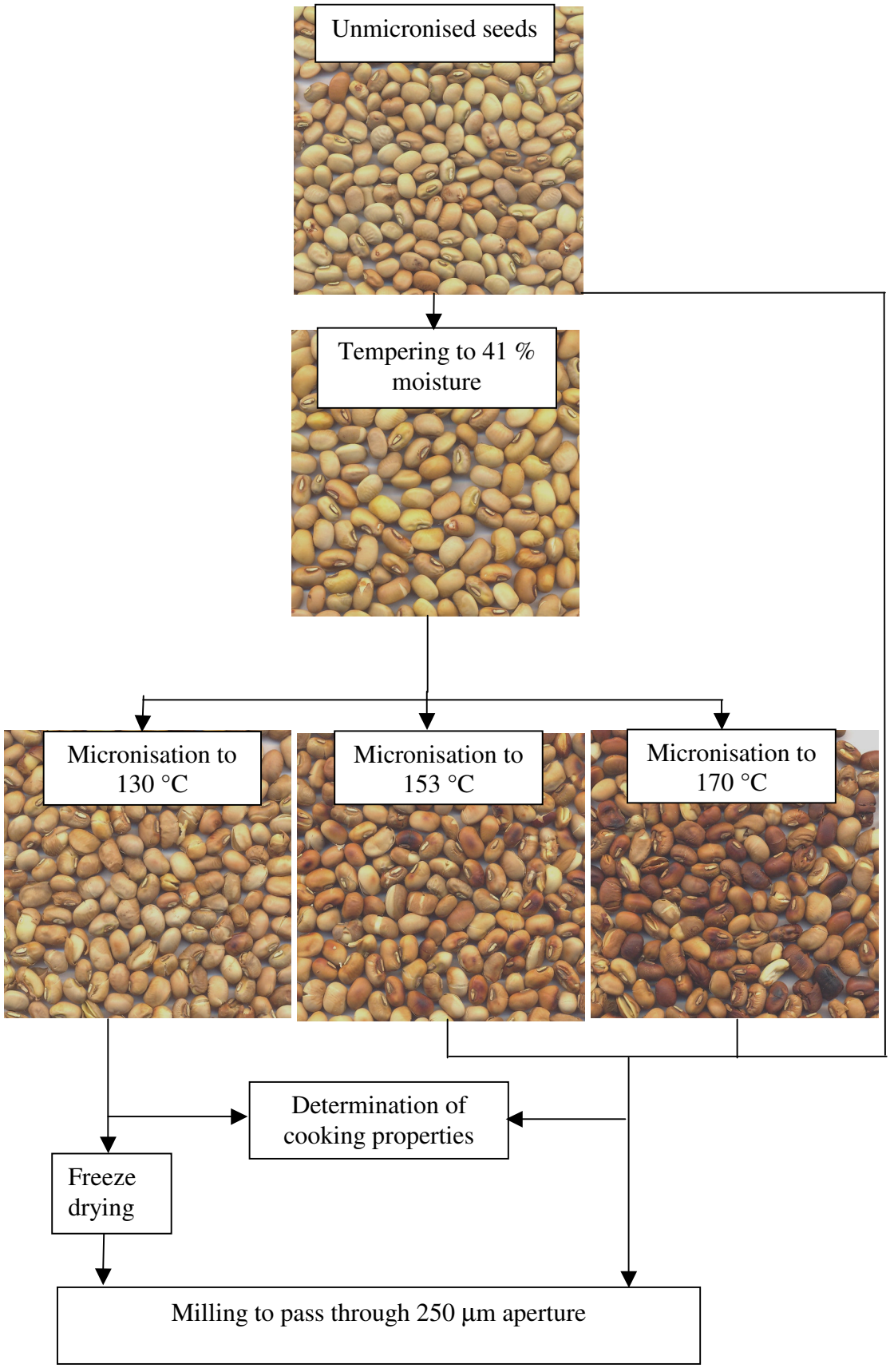

Figure 3.3.1 Experimental flow diagram

3.3.2.5 Determination of water absorption during cooking

The amount of water absorbed during cooking was determined according to a modified method of Cenkowski and Sosulski (1997) as reported in section 3.1.2.5.

3.3.2.6 Splitting of cowpea seeds during cooking

The tendency of seeds to split during cooking was determined according to the method of van Buren *et al*. (1986) as reported in section 3.1.2.6.

3.3.2.7 Texture of cowpea seeds during cooking

Texture was measured as work (area under the curve, Nmm) done to cut individual cowpea seeds through a distance of 5 mm at a speed of 4 mm/s based on the method of Sefa-Dedeh *et al*. (1978) as reported in section 3.2.2.11.

3.3.2.8 Determination of cooking time

A Mattson bean cooker (custom made) as described by Wang *et al*. (2003) was used to determine the cooking time of micronised (41 % moisture, 130, 153 and 170 °C) and unmicronised cowpea samples as reported in section 3.2.2.12.

3.3.2.9 Cowpea flour preparation

The cowpea samples (41 % moisture) micronised to 130 °C were freeze dried before milling. Raw and micronised (41 % moisture, 130, 153 and 170 °C) cowpeas were milled (Falling number mill, 3100) to pass through a 250 µm-aperture sieve. The milled flour samples were vacuum-sealed and stored at 4 ºC until they were used to determine total starch, enzyme-susceptible starch, amylose content, pasting and thermal properties.

3.3.2.10 Determination of total and enzyme-susceptible starch

Total starch content of the unmicronised and micronised (M-130 °C, M-153 °C and M-170 °C) cowpea flour was determined according to the AOAC method 996.11 using the Megazyme total starch assay kit (Megazyme, Wicklow, Ireland) (McCleary *et al*., 1997). Flour samples (0.1 g) of unmicronised and micronised (M-130 °C, M-153 °C and M-170 °C) cowpeas were vortexed in 0.2 ml aqueous ethanol (80 % v/v) and digested by 300 units of thermostable α-amylase (in 3 ml of 50 mM MOPS buffer, pH 7) in a boiling water bath (96 °C) for 6 min with intermittent stirring. The

samples were cooled and 4 ml of 200 mM sodium acetate buffer (pH 4.5) was added followed by 30 min digestion using 20 units of amyloglucosidase (0.1 ml) at 50 °C. The method was modified slightly to determine enzyme-susceptible starch (ESS) by digesting the samples with thermo stable α-amylase at 37 °C. The hydrolysed glucose was determined using the glucose oxidase peroxidase (GOPOD) assay.

3.3.2.11 Determination of digestible amylose

The digestible amylose content of the cowpea flour was determined according to the Con A method described by Gibson, Solah & McCleary, (1997) using the Megazyme amylose/amylopectin assay kit (Megazyme, Wicklow, Ireland).

3.3.2.12 Determination of carbohydrate solubility using size exclusion HPLC-Gel permeation chromatography

HPLC-Gel Permeation (HPLC-GP) chromatography was performed using three Shodex lonpax S-800 series styrenedivinylbenzene columns connected in series (Showa Denko K.K., Tokyo, Japan) (Jackson, Waniska & Rooney, 1989). The column packing was a strong sodium cation-exchange resin, designed for the separation of saccharides. The three columns were connected in the order of S-805/S (estimated exclusion limit (EEL) = 5 x 106 MW), S-804/S (EEL = 5 x 105 MW), and S-803/S (EEL = 5 x 104 MW). An oven maintained the column temperature at 50 ºC. The mobile phase was deionised water, HPLC-grade, which was pumped with a Beckman-110B HPLC pump operating at 1.0 ml min^{-1} (Beckman Instrument Inc., Berkeley, CA.). The columns were connected to a Waters model 410 refractive index detector (Millipore Corporation, Milford, MA). The refraction index cell was maintained at 50 ºC. Pullulan standards (MW 5300-853000) were utilised to identify the molecular weight (MW) of the eluant. Soluble amylopectin, amylose, oligosaccharides and sugars of unmicronised and M-170 °C flour were extracted in water for 10 min at 98 °C and quantified in the HPLC-GP system.

3.3.2.13 Enzyme treatment and light microscopy of cowpea flour dispersions

Enzyme digestion of cowpea flour was conducted in order to identify the different macromolecules involved in reduced pasting viscosity of the M-170 °C cowpea flour. The digestion process was based on the method reported by Zhou, Hoover & Liu (2004). Cowpea flour was sequentially digested with α-amylase followed by protease

and the remaining undigested material was split in half; one half was digested with α- amylase and the other part with pectinase. Cowpea flour (0.6 g) was mixed with 4 ml α-amylase (Megazyme International, Wicklow, Ireland) in 30 ml pH 7 MOPS (3-N-Morpholinopropanesulphonic acid) buffer and digested at 37 ºC for 16 h. The slurry was centrifuged 1000 *x g* for 5 min and the supernatant was discarded. The sediment was washed in 15 ml of water followed by centrifugation. A sample of the sediment was taken and mounted on a glass slide and protein was stained for 5 min with 1 drop of Acid Fuchsin (1 % w/v in water; Fisher Scientific, USA) while Congo Red (1 % w/v in water; Fisher Scientific, USA) stained for glucans. The remaining sediment was digested with 10 ml protease (1g/ml, Sigma EC 232-752-2) in pH 7.5 phosphate buffer for 1 h at 37 °C. The sample was centrifuged and the supernatant was discarded followed by washing with water. A sample of the sediment was mounted on a glass slide as described before. The remaining sediment was split into two fractions. One fraction was digested for 1 h in 10 ml α-amylase solution. The pH of the remaining fraction was adjusted to 4.5 using 25 % acetic acid followed by 1 h digestion with 2 ml of pectinase (Pectinase from Rhizopus Sp, CAS No. 9032-75-1, Sigma Inc, St Louis Missouri) at room temperature. The remaining sediments were mounted on glass slides. Photomicrographs of the mounted samples were taken using a Nikon Coolpix 995 camera (Nikon USA) mounted on a Zeiss Universal bright field microscope equipped with polarising filters. Samples were viewed with polarisers to determine the extent of birefringence and without polarisers to view the extent of staining for components.

3.3.2.14 Isolation of starch from micronised (41 % moisture, 130 °C and 170 °C) cowpeas

Starch from unmicronised and micronised (41 % moisture, 130 °C and 170 °C) cowpeas was isolated based on the method of Taylor, Novellie & Liebenberg (1984). Cowpea starch was isolated from raw and micronised (41 % moisture, 130 and 170 °C) cowpeas milled to pass through a sieve with aperture size 250 µm. The flour (100 g) was mixed with 500 ml deionised water and stirred at 22 °C for 2 h. The slurry was wet milled using a Retsch wet mill (Monitoring and Control Laboratories, Johannesburg, South Africa) and filtered through sieves (Labotech, Johannesburg, South Africa) with aperture sizes 212, 108, 75 and 45 µm on to a collecting pan. The sediment on the sieve was suspended in distilled water and wet milled and the

resulting slurry filtered through the sieves. The wet milling process was repeated until the filtrate was clear. The filtrate was centrifuged at 3880-x *g* for 5 min (15 °C). The supernatant was carefully decanted and the sediment was re-suspended in deionised water and vigorously mixed. The mixture was centrifuged at 3880 x *g* for 5 min (15 °C) and the supernatant liquid was decanted carefully. The brown layer on top of the starch sediment was carefully scooped out. The remaining starch sediment was re-suspended in deionised water and centrifuged. The washing, removing of the brown layer, suspension in water and centrifuging was repeated for 6 to 7 times. The starch was then washed with hexane to remove fat residues followed by drying at 37 °C for 48 h. The dried starch was ground with a motor and pestle to pass through a sieve with aperture size 109 µm.

3.3.2.15 Thermal analysis of cowpea flour and extracted starch

Thermal properties of flour from unmicronised and micronised (41 % moisture, 130 and 170 °C) cowpea seeds were measured according to the method reported by Ji, Seetharaman, Wong, Pollack, Duvick, Jane & White (2003). Cowpea flour samples were mixed with water in the ratio of 2:1 and heated in a sealed stainless steel pan. A Perkin-Elmer DSC-7 analyzer (Norwalk, CT) equipped with thermal analysis software (Perkin-Elmer Corporation, Norwalk, CT) was used to scan the samples at a rate of 10 ºC min^{-1} from 30 to 110 ºC. All measurements were carried out on duplicate samples. The following thermal parameters were measured: the melting enthalpy (Δ H) in J g^{-1}, peak onset (To), peak (Tp) and peak end (Tc) temperatures. The degree of starch conversion was calculated as: 100 - % decrease in enthalpy as a result of the heat treatment.

3.3.2.16 Pasting properties of cowpea flour and isolated starch

Pasting properties of cowpea flour and starch isolated from unmicronised and micronised (41 % moisture, 130 and 170 °C) samples were determined using a rapid visco-analyser's (Newport Scientific Pty Ltd, Warriewood, Australia) standard profile 2 with modifications to allow for lower boiling point (96 °C). Suspensions of flour (3.5 g) on a dry basis were prepared in 25 ml of deionised water. The suspension was equilibrated at 50 ºC for 1 min followed by programmed heating to 91 ºC at a uniform rate of 5 ºC min^{-1} with constant stirring at 160 rpm. The heated slurry was held at

91 ºC for 7 min then cooled to 50 ºC at the same rate and held at this temperature for 2 min.

3.3.2.17 Statistical analysis

The experimental assays were repeated three times to generate data that was analysed using Statistica (StatSoft, Inc., Tulsa, OK) statistical software version 6.0. Analysis of variance (ANOVA) was used to evaluate the data based on a 5 % level of significance. When the F-test was significant, differences between means were determined using the least significant difference (LSD) test.

3.3.3 Results and discussion

Following the micronisation process (41 % moisture and Infrared heating), the M-130 °C cowpeas had significantly ($P \leq 0.05$) higher moisture content than the unmicronised and the other micronisation temperatures (Table 3.3.1). As such, cowpeas micronised to 130 °C would require additional drying to achieve shelf stability (Table 3.3.1). Increasing the micronisation temperature to 153 and 170 ºC reduced the moisture content to levels adequate for shelf stability.

Cooking of dry leguminous seeds involves seed hydration and heating. Dry seeds are sometimes hydrated by soaking in water prior to cooking. The length of soaking time depends on hydration properties of the seeds (Sefa-Dedeh *et al*., (1978); Sefa-Dedeh & Stanley (1979a). Micronised (41 % moisture, 130, 153 and 170 ºC) cowpeas had a higher rate of water absorption and absorbed more ($P \leq 0.05$) water during 6 h of soaking at 22 ºC than the unmicronised cowpeas (Figure 3.3.2). Water absorption during soaking of legume seeds is related to physical and chemical properties of the seed, such as seed coat properties, starch, protein (Sefa-Dedeh & Stanley, 1979a), and density as explained in section 3.2.3.1. It was observed that micronised (41 % moisture, 153 °C) cowpea seeds were less dense than unmicronised cowpea seeds and showed fissured seed coat and cotyledon, which contributed towards increased water uptake during soaking.

Following 3 h of soaking, M-153 °C and M-170 °C cowpeas absorbed significantly ($P \leq 0.05$) more water than the M-130 °C cowpeas (Figure 3.3.2). The difference in water absorption pattern during soaking (22 °C) was probably due to the differences in

moisture content following the micronisation treatment (Table 3.3.1). A positive correlation has been reported between the initial moisture content of legume seeds and the rate of water uptake during the 1st hour of soaking (Moscoso, Bourne & Hood, 1984).

Table 3.3.1 Effect of micronisation temperature (130, 153 and 170 °C) on some physicochemical characteristics of moisture-conditioned (41 %) cowpeas

Physicochemical characteristic	[y]Unmicronised	[z]Micronisation temperature (°C)		
		130	153	170
Moisture (g kg^{-1})	89^{c} (4)	252^{a} (18)	121^{b} (22)	50^{d}(9)
Cooking time (min)	57^{a} (2)	30^{c} (5)	32^{c} (7)	39^{b} (2)
Water absorbed (g kg^{-1})				
30 min of cooking	875^{d} (45)	1189^{c} (36)	1393^{b} (45)	1487^{a} (37)
60 min of cooking	1538^{b} (31)	1419^{c} (39)	1713^{a} (39)	1667^{a} (50)
Texture (Work) (Nmm)				
30 min of cooking	6.9^{a} (1.6)	4.4^{b} (1.0)	4.7^{b} (1.1)	4.4^{b} (0.9)
60 min of cooking	3.2^{a} (0.9)	3.1^{a} (0.8)	2.6^{b} (0.9)	3.0^{a} (0.4)
Splits (%)				
30 min of cooking	3^{b} (1.6)	40^{a} (8.4)	44^{a} (7.1)	42^{a} (1.6)
60 min of cooking	45^{c} (10.0)	74^{a} (10.1)	62^{b} (7.6)	48^{c} (4.6)
Total starch (g kg^{-1})	445 (17.2)	452 (13.3)	459 (17.3)	473 (15.6)
ESS (%)	21^{c} (2.7)	73^{a} (2.8)	68^{b} (2.8)	68^{b} (1.8)
Digestible amylose (%)	19.5^{a} (1.8)	12.7^{b} (3.3)	15.1^{b} (2.3)	14.9^{b} (1.7)

ESS= Enzyme-susceptible starch; [y]Unmicronised = Raw, [z]Micronisation temperature (°C) = Temperature for micronisation of moisture-conditioned seeds (41 %); means followed by the same letter within a row are not significantly different at level $P \leq 0.05$; standard deviations of the means are in parenthesis

3.3.3.1 Cooking characteristics of cowpeas

Cooking time is one of the cooking characteristics of leguminous grains that is widely evaluated, since long cooking time seems to be one of the main factors contributing to the dismal utilisation of legumes such as cowpeas (Taiwo, 1998).

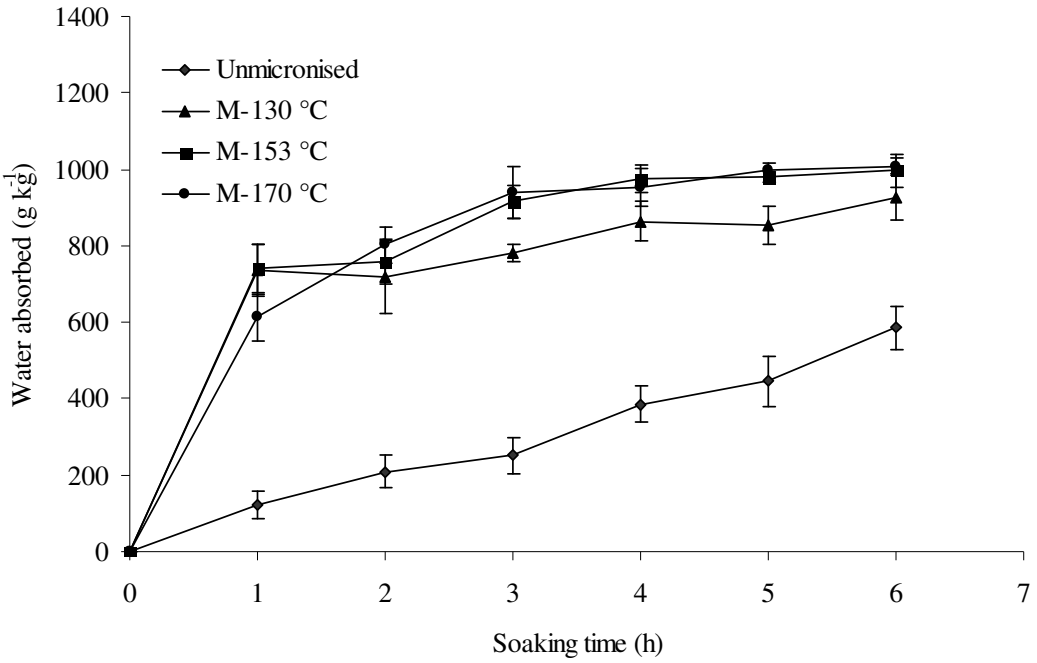

Figure 3.3.2 Effect of micronisation temperature (130 °C, 153 °C and 170 °C) on water absorption during 6 h of soaking (22 °C) Bechuana white cowpeas (vertical bars indicate standard deviations of the means Unmicronised= Raw, M= Micronised (41 % moisture, 130 °C, 153 °C and 170 °C))

The unmicronised Bechuana white cowpeas had a cooking time of 57 min, which was significantly ($P \leq 0.05$), reduced by 47, 44 and 32 % following micronisation (41 % moisture-conditioning and infrared heating) to 130, 153 and 170 °C, respectively (Table 3.3.1). Similar reductions in cooking time of moisture-conditioned and micronised legumes have been reported for lentils (Arntfield *et al*., 1997; Cenkowski & Sosulski, 1997; Arntfield *et al*., 2001), split peas (Cenkowski & Sosulski, 1998) and beans (Bellido *et al*., 2006). Cooking time in legumes refers to the time required

for seeds to attain a texture that is acceptable for consumption (Arntfield *et al*., 2001). It is evident that unmicronised cowpeas required a significantly ($P \leq 0.05$) longer cooking time than the three micronised (41 % moisture, 130, 153 and 170 °C) samples in order to attain a soft texture (Figure 3.3.3; Table 3.3.1). This is consistent with other research work on moisture-conditioned and micronised legumes (Arntfield *et al*., 1997; Cenkowski & Sosulski, 1997; Arntfield *et al*., 2001; Bellido *et al*., 2006). The softening of dry legume seed texture during cooking has mainly been ascribed to parenchyma cell separation along the middle lamella (Sefa-Dedeh *et al*., 1978) due to β elimination of pectic substances (Liu *et al*., 1993b; Coultate, 2002) and starch gelatinisation within the cotyledon parenchyma cell (Sefa-Dedeh *et al*., 1979).

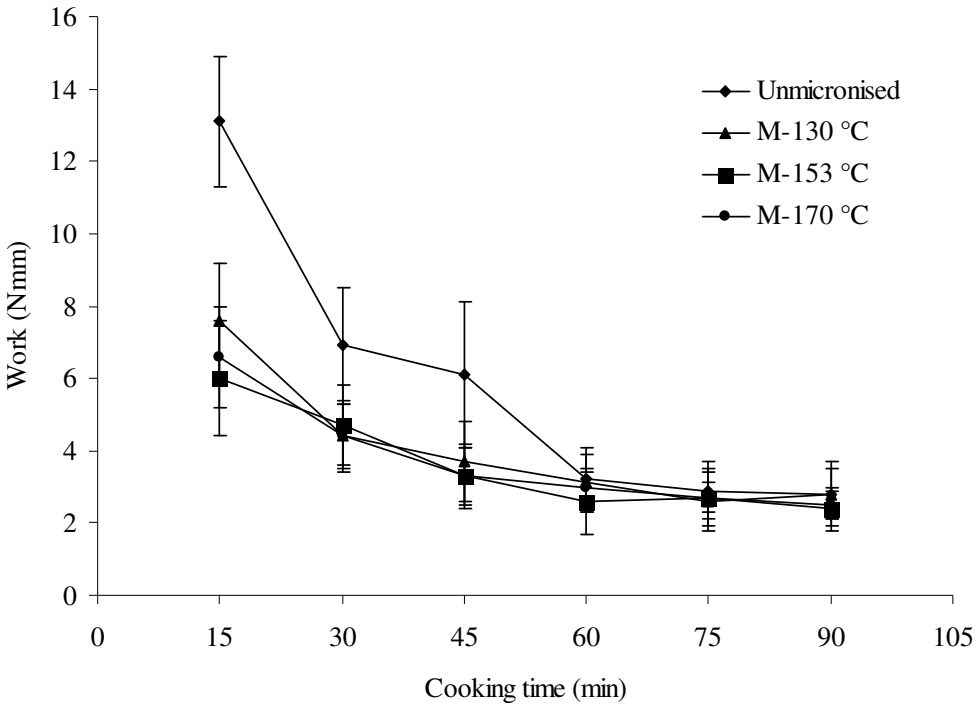

Figure 3.3.3 Effect of micronisation temperature (130 °C, 153 °C and 170 °C) on texture (Work, N mm) during 90 min of cooking Bechuana white cowpeas (vertical bars indicate standard deviations of the means Unmicronised= Raw, M= Micronised (41 % moisture, 130 °C, 153 °C and 170 °C))

Since β elimination of pectic substances and starch gelatinisation require water, improved water uptake during cooking of legumes has been related with reduction in cooking time (Cenkowski & Sosulski, 1997; Phadi, 2004). A significant ($P \leq 0.05$) negative correlation ($r = -0.90$) existed between water absorption during cooking and texture (work required to cut through a seed). Micronisation (41 % moisture, 130, 153 and 170 °C) significantly improved the amount of water absorbed by the cowpeas during the first 45 min of cooking (Table 3.3.1, Figure 3.3.4) possibly due to reduced bulk density and development of fissures as reported in section 3.2.3.2.

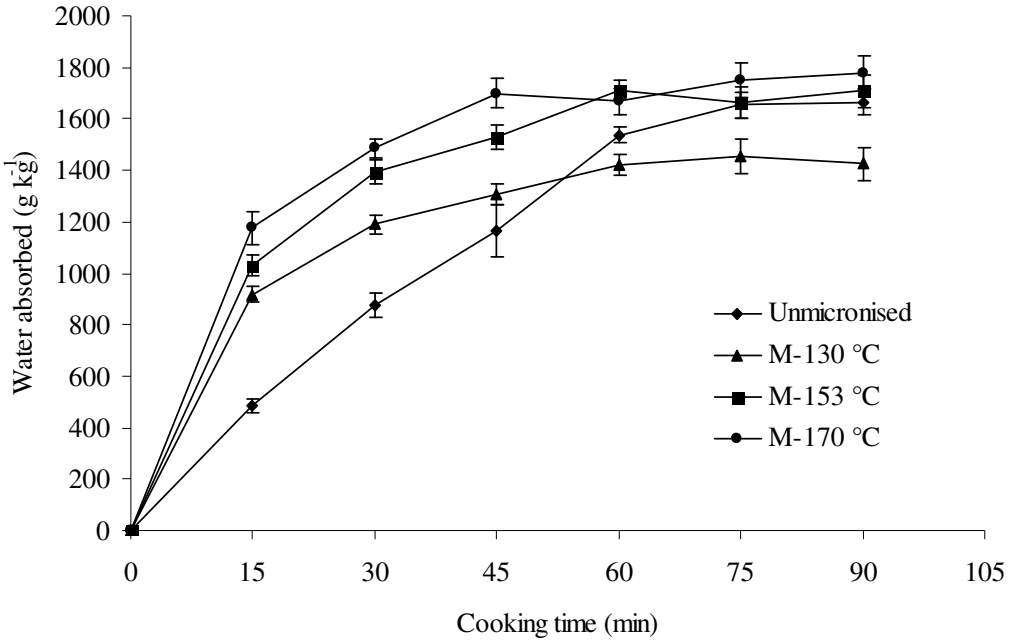

Figure 3.3.4 Effect of micronisation temperature (130 °C, 153 °C and 170 °C) on water absorption during 90 min of cooking Bechuana white cowpeas; (vertical bars indicate standard deviations of the means Unmicronised= Raw, M= Micronised (41 % moisture, 130 °C, 153 °C and 170 °C))

Micronised (41 % moisture, 153 °C) cowpea seeds as reported in Section 3.2 to have reduced bulk density in comparison to unmicronised seeds. The reduction in bulk

density implies that the micronised (41 % moisture, 130, 153 and 170 °C) seeds have air spaces within the cotyledon, which would enhance water uptake.

Pressure build up within the seeds during micronisation gives rise to the development of fissures in the seed coat, cotyledon and cell wall of micronised (41 % moisture, 153 °C) cowpeas seeds. These fissures contribute towards improved water absorption by the seeds during cooking. In addition, the fissuring of the cotyledon may also have led to significant ($P \leq 0.05$) increase in the incidence of splitting in micronised (130, 153 and 170 °C) cowpeas when cooked for 30 min (Table 3.3.1, Figure 3.3.5). The level of splitting in micronised (41 % moisture, 130, 153 and 170 °C) samples at 30 min of cooking was not significantly ($P \leq 0.05$) different to the level of splitting for unmicronised cowpeas cooked for 60 min. Since the cooking time of M-130 °C, M-153 °C and M-170 °C cowpeas was 30, 32 and 39 min respectively (Table 3.3.1), it means that effectively there is no difference in splitting of the cooked samples.

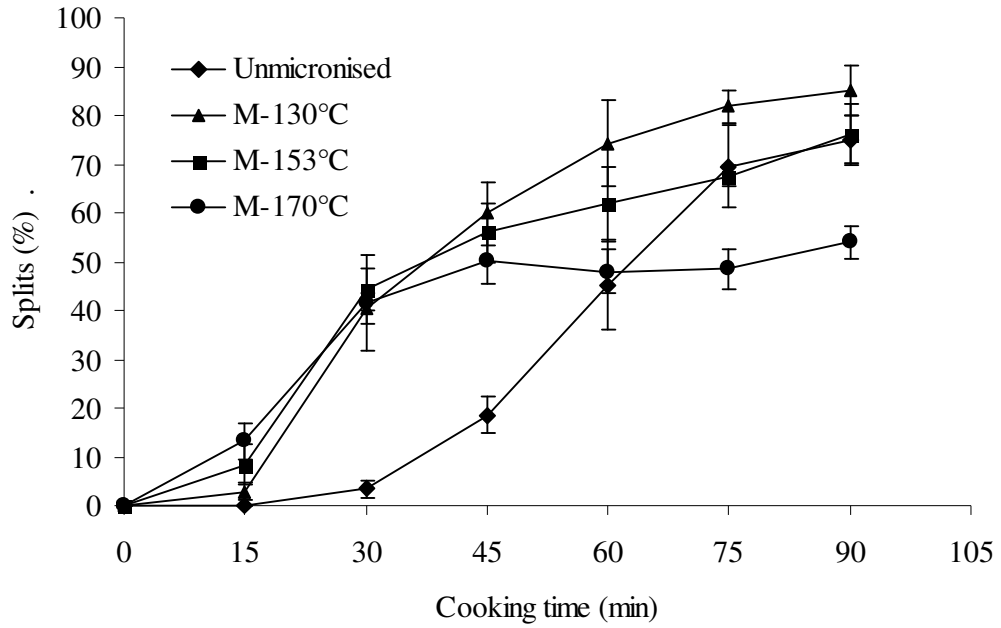

Figure 3.3.5 Effect of micronisation temperature (130 °C, 153 °C and 170 °C) on splitting (%) during 90 min of cooking Bechuana white cowpeas, (vertical bars indicate standard deviations of the means Unmicronised= Raw, M= Micronised (41 % moisture, 130 °C, 153 °C and 170 °C))

Splitting of unmicronised, M-130 ºC and M-153 °C cowpeas increased with extended cooking time, while there was no significant increase in splitting after 30 min of cooking M-170 °C cowpeas. It has been postulated that the incidence of splitting during cooking of unmicronised cowpea seeds is positively related to increase in water absorption during cooking (Taiwo, 1998). Although the M-170 °C cowpeas had high water absorption throughout the cooking process comparable with unmicronised, M-130 °C and 153 °C cowpeas, this was not accompanied with increased incidence of splitting during extended cooking. This reduction in splitting during extended cooking shows that micronisation to a higher temperature possibly resulted in a cotyledon structure that did not easily disintegrate during extended cooking.

Overall, the two lower micronisation temperatures (130 and 153 °C) were more effective in reducing cooking time of cowpeas than the higher temperature of 170 °C (Table 3.3.1). The longer ($P \leq 0.05$) cooking time and splitting pattern observed in M-170 °C cowpeas could possibly be due to differences in the extent of starch modification during micronisation involving higher temperatures.

3.3.3.2 Effect of micronisation temperature of cowpea seeds on starch-related properties

Since there were no significant differences between the cooking characteristics of M-130 °C and M-153 °C cowpeas, some of the subsequent work on starch was conducted using M-130 °C and M-170 °C cowpeas to represent a mild and intense heat treatment. Starch gelatinisation is a complex process, where the structure and functionality of the end product varies depending on process conditions such as temperature, moisture as well as the presence or the absence of shear (Tananuwong & Reid, 2004; van de Einde, Akkermans, van der Goot & Boom, 2004). The micronisation (130 and 170 °C) of cowpeas with 41 % moisture changed the crystalline order and nature of the cowpea starch granules. This is evidenced by reduced transitional enthalpies (Table 3.3.2), loss of birefringence and increased susceptibility to α-amylase digestion (Table 3.3.1).

Cowpea flour milled from cowpeas micronised to 130 and 170 °C seeds had higher transition onset (To) and peak (Tp) temperatures (Table 3.3.2). Enthalpies of cowpea flours were much lower than that of the starch isolated from the samples; however,

thermal transition temperatures of the isolated starch were lower compared to flour samples (Table 3.3.2). Transitional enthalpies for heterogeneous systems such as cowpeas flour are a composite of energy transitions in the system, which includes starch gelatinisation and protein denaturation (Henshaw *et al*., 2003). The starch isolated from micronised (41 % moisture, 130 and 170 °C) samples also exhibited higher gelatinisation and onset temperatures (To). Similar thermal transitions for cowpea flour have been reported earlier (Henshaw *et al*., 2003).

Table 3.3.2 Effect of micronisation temperature on thermal properties of cowpea flour and isolated starch

Thermal properties	[y]Unmicronised	[z]Micronisation temperature (°C)		
		130	153	170
Cowpea flour				
Onset To (ºC)	70.5[c] (0.1)	74.8[b] (1.2)	78.9 [a] (0.9)	74.4[b] (0.4)
Peak (ºC)	78.0[c] (1.0)	80.8 [bc] (0.2)	83.6[ab] (2.1)	84.4[a] (0.4)
Tc (ºC)	85.7 [b] (1.4)	93.5[ab] (2.2)	94.3[a] (7.9)	95.6 [a] (3.9)
Enthalpy (Δ H, J/g sample)	2.5 (0.6)	4.02 (2.1)	2.2	1.8 (0.8)
Cowpea starch				
Isolated starch yield (%)	66	44	ND	11
Onset To (ºC)	62.7 [b] (0.6)	68.0 [a] (1.3)	ND	69.8 [a] (0.3)
Peak (ºC)	72.7 [b] (0.4)	76.4 [a] (0.2)	ND	77.3 [a] (0.7)
Tc (ºC)	87.3 (3.3)	97.2 (4.7)	ND	93.7 (2.1)
Enthalpy (Δ H, J/g)	12.9 (1.8)	11.9 (4.0)	ND	5.5 (0.6)
Change in enthalpy (%)		8	ND	57

[y]Unmicronised = Raw, [z]Micronisation temperature (°C) = Temperature for micronisation of moisture-conditioned seeds (41 %); Means followed by the same letter within a row are not significantly different at level $P \leq 0.05$; standard deviations of the means are in parenthesis; ND = Not determined

The higher enthalpies observed in starch isolated from micronised (41 % moisture, 130 and 170 °C) samples could possibly be due to non-homogeneous thermal treatment among cowpeas and/or within the cowpea which was characterised by the reduced yields of isolated starch from micronised (41 % moisture, 130, and 170 °C) cowpeas. This contributed to the higher standard deviations in transitional enthalpies for these samples (Table 3.3.2). When the starch granules from the micronised (41 % moisture, 130 and 170 °C) seeds were observed under polarised light (Figure 3.3.6 (2b) and 3.3.6 (3b)), they did not exhibit birefringence.

Light microscopy of α-amylase digested flours from unmicronised and micronised (41 % moisture, 130 and 170 °C) cowpeas revealed a reduction in the amount of starch granules in micronised (41 % moisture, 130 and 170 °C) samples. This confirmed that the modified starch granules from micronised seeds (41 % moisture, 130 and 170 °C) were more susceptible to α-amylase digestion (Table 3.3.1) than native starch granules of unmicronised flour. These results indicate that micronisation of moisture-conditioned cowpea seeds altered the granular structure of cowpea starch thereby requiring less time to hydrate and disrupt the remaining starch structure during subsequent cooking. Cenkowski and Sosulski (1997) used DSC analysis of lentil flours, to demonstrate that 5 min of cooking was adequate to fully gelatinise the starch in micronised (38.6 % moisture, 150 °C) lentils as compared to 50 % of the starch for untreated lentils. Gelatinisation of starch during cooking of legumes is a very important phenomenon that has a positive correlation with texture of cooked seeds (Arntfield *et al*., 1997).

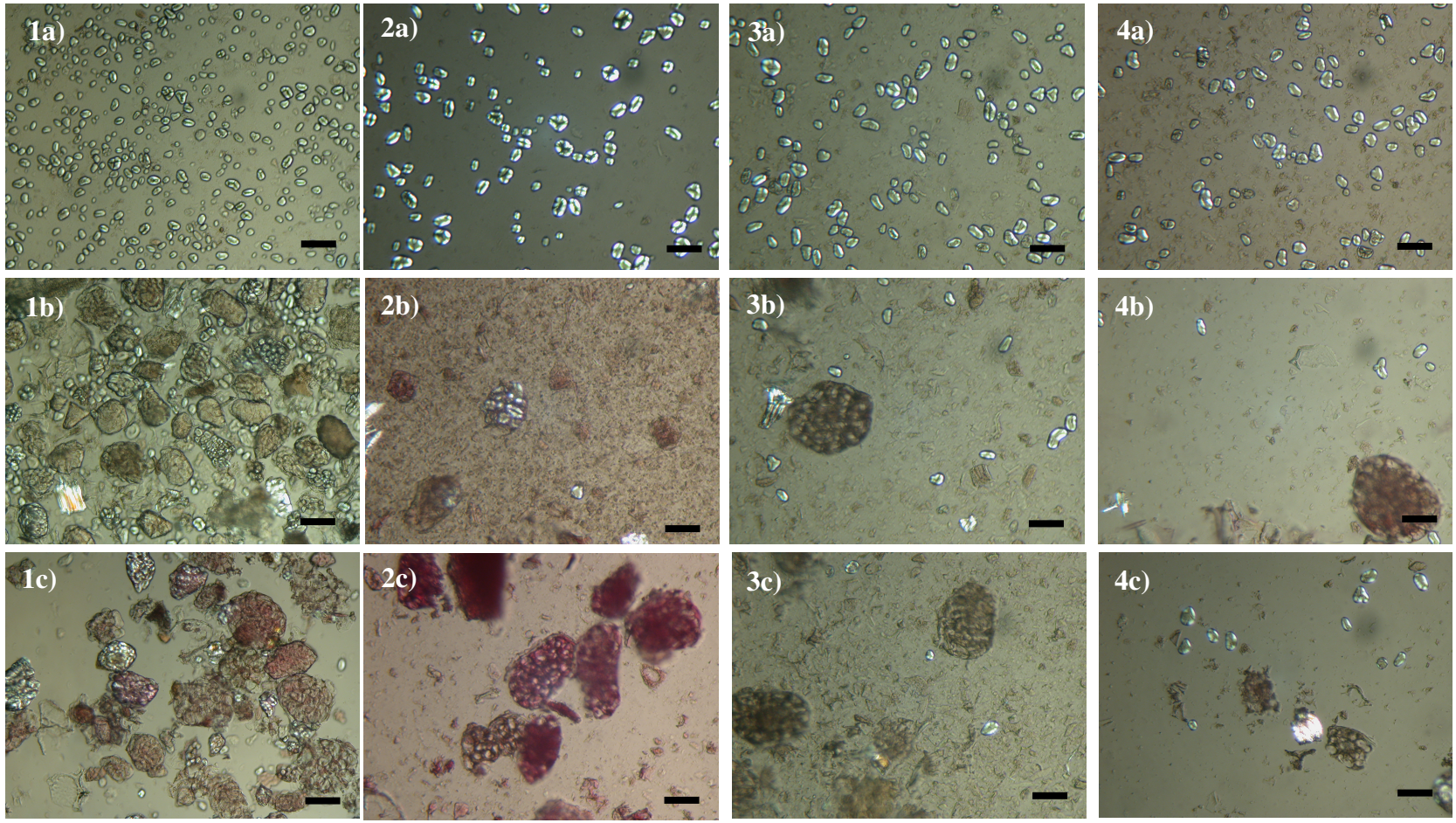

Figure 3.3.6 **Effect of micronisation temperatures on cowpea flour from micronised (41 % moisture, 130 °C and 170°C) seeds during sequential enzyme digestion (stained with acid Fuchsin): 1 = No enzyme treatment, 2 = α –amylase, 3 = α –amylase - protease, and 4 = α –amylase-protease- α –amylase; a = unmicronised, b = M-130 ºC and c = M-170 ºC, Bar ~50 nm**

Pasting curves have been used to track starch granule swelling and stability of native and hydrothermally treated cowpea flours (Prinyawiwatkul *et al*., 1997c; Henshaw *et al*., 2002). The flour from unmicronised cowpeas exhibited a characteristic pasting curve (Figure 3.3.7), which was comparable with patterns reported for cowpea flours (Prinyawiwatkul *et al*., 1997c; Henshaw *et al*., 2002). However, flour from micronised seeds (41 % moisture, 130 and 170 °C) did not give the characteristic cold swelling peak expected in pregelatinised starch (Lai, 2001) (Figure 3.3.7).

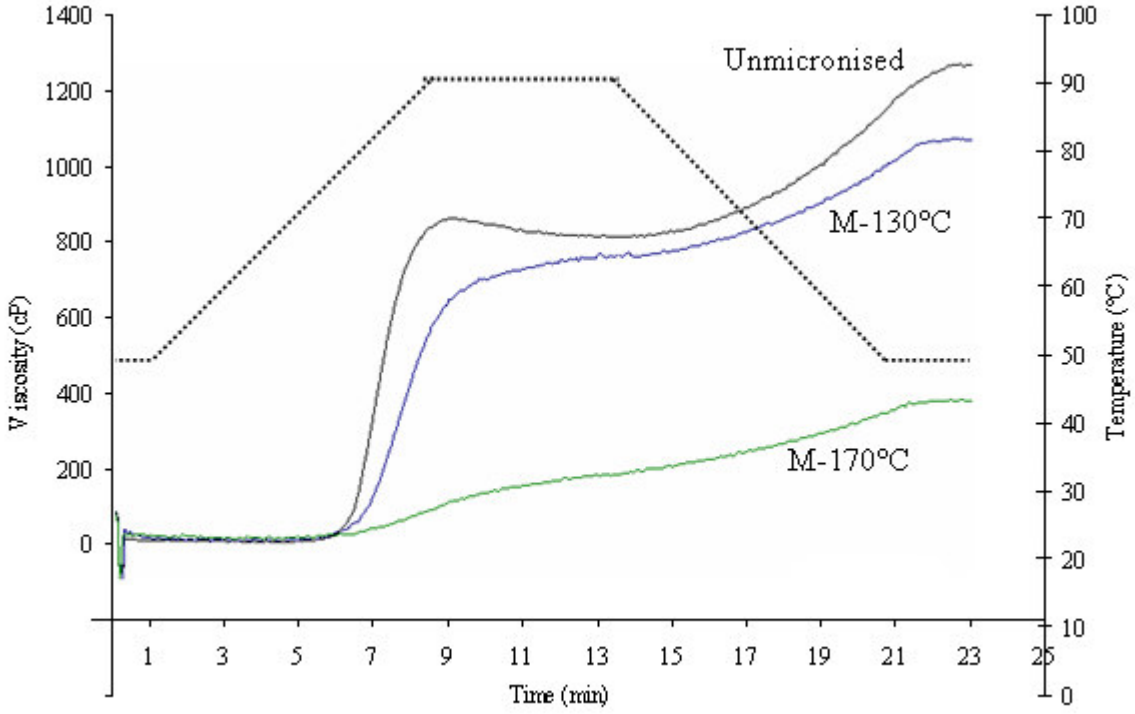

Figure 3.3.7 **Effect of micronisation temperature on pasting properties of cowpea flour from micronised (41 % moisture, 130 °C and 170 °C) seeds, (Unmicronised= Raw, M= Micronised (41 % moisture, 130 and 170 °C))**

Some of the starch granules were imbedded in the denatured protein matrix within the parenchyma cells (Figures 3.3.6 (2a) and 3.3.6 (3a)), which could reduce water access. Similarly, Cenkowski and Sosulski (1997) indicated the absence of a cold swelling peak for flour from micronised (26 % moisture; 110 °C) split peas. Prinyawiwatkul *et al.* (1997c) also illustrated that flour from cowpeas that had been soaked (24 h) and boiled (45 min) did not exhibit cold swelling peak.

The pasting curve for the M-130 °C flour shifted towards higher temperatures ($P \leq 0.05$) (Figure 3.3.7). Similar results have also been reported in microwave treated lentil starch (Gonzalez & Perez, 2002). Increase in pasting temperature of hydrothermally treated starch has been related to granule modification that limits water access into the granule (Hoover & Manuel, 1996a) and the presence of other competing hydrophilic molecules such as protein. The increase in pasting temperature is consistent with results on thermal properties of the cowpea flours which showed a 4 °C increase in transition onset temperature (To) for the M-130 ºC cowpea flour (Table 3.3.2). In hydrothermally treated starches, increase in onset temperature (To) is mainly attributed to amylose-amylose interactions that lead to increased starch granule crystallinity requiring higher temperatures to be melted (Hoover & Manuel, 1996a; Hoover & Manuel, 1996b). Concomitantly, micronisation (41 % moisture, 130 °C and 170 °C) significantly reduced the amount of digestible amylose of the cowpea flour (Table 3.3.1). Thus the reduction in digestible amylose could possibly be due to the formation of amylose-lipid complexes (Hoover & Manuel, 1996a; Hoover & Manuel, 1996b) and/or re-crystallisation of dispersed amylose during retrogradation of gelatinised starch (Arntfield *et al.*, 1997).

In addition there was a significant ($P \leq 0.05$) progressive decline in pasting viscosities of the flours with increase in micronisation temperature (Figure 3.3.7). The flour from M-130 ºC cowpeas had lower ($P \leq 0.05$) peak viscosity than the flour from unmicronised seeds, while there was a severe ($P \leq 0.05$) reduction in flour paste viscosity for the M-170 ºC flour (Figure 3.3.7). This is in contrast to the results obtained by Cenkowski and Sosulski (1998) where an increase in starch swelling was observed.

When high amylose starch is exposed to a hydrothermal treatment, the amylose molecules in the granule can realign through hydrogen bonding involving other amylose, amylopectin and/or lipids (Hoover & Manuel, 1996a). These associations may result in physically induced crosslinked starch, which has reduced swelling potential during subsequent gelatinisation. The possible existence of such crosslinks involving starch in moisture-conditioned and micronised cowpeas in this research was studied through enzymatic digestion and HPLC-GP. Retrograded starch is resistant to α-amylase hydrolysis and is referred to as resistant starch type-III (Karim, Norziah, & Seow, 2000). Micronisation (41 % moisture, 130, 153 170 ºC) significantly reduced the level of digestible amylose (Table 3.3.1).

HPLC-GP analysis of the samples showed that micronisation of moisture-conditioned seeds to 170 °C did not have a significant ($P \leq 0.05$) effect on the solubility of amylopectin; however the treatment significantly ($P \leq 0.05$) reduced the amylose solubility from 11.6 to 8.8 % (Figure 3.3.8). The reduced solubility for amylose at 98 °C is possibly due to amylose-amylose associations formed during starch retrogradation. Amylose-amylose associations have a higher melting point (in the range of 120-170 °C) than amylose-amylopectin associations (40-100 °C) (Karim *et al*., 2000) and hence could not have been dispersed into solution at the temperature (98 °C) used in this study. Hoover and Manuel (1996a) reported similar decrease in amylose leaching following heat moisture treatment of legume starches.

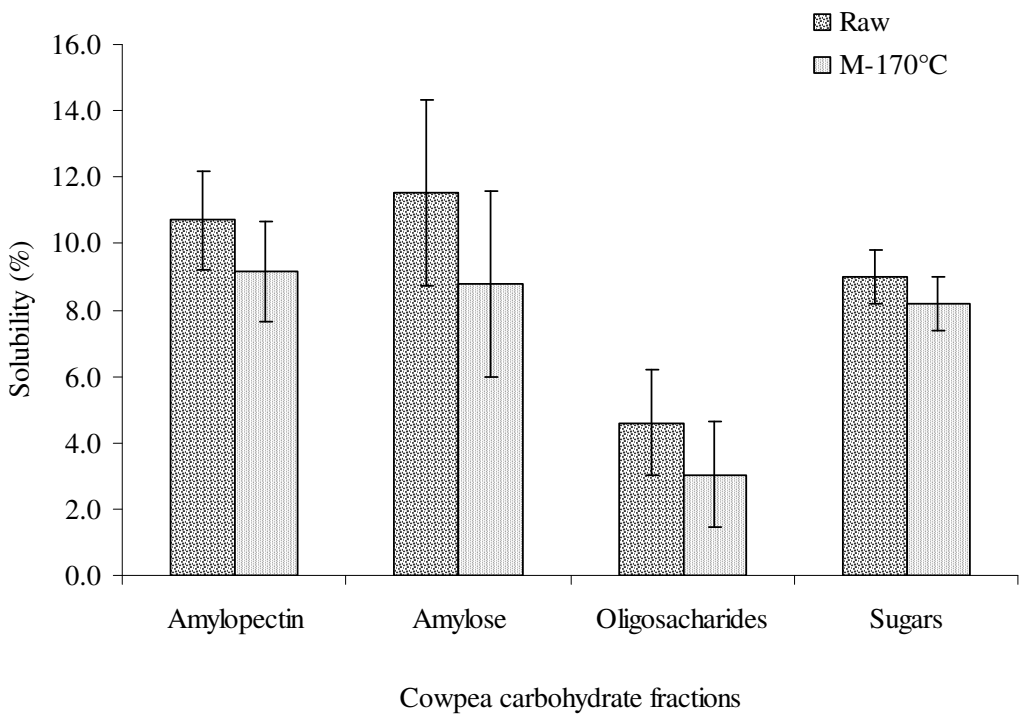

Figure 3.3.8 Effect of micronisation temperature of 170 °C on solubility of amylopectin, amylose, oligosaccharides and sugars from micronised (41 % moisture 170 °C) seeds, (vertical bars indicate standard deviations of the means M - 170 °C = Micronised (41 % moisture 170 °C))

The paste viscosity for the M-170 °C flour gradually increased throughout the heating, holding and cooling periods without forming a peak. The gradual increase in viscosity during the pasting process could be ascribed to the gelatinisation of residual starch granules (Figure 3.3.9), which comprised approximately 11 % of the total cowpea starch content.

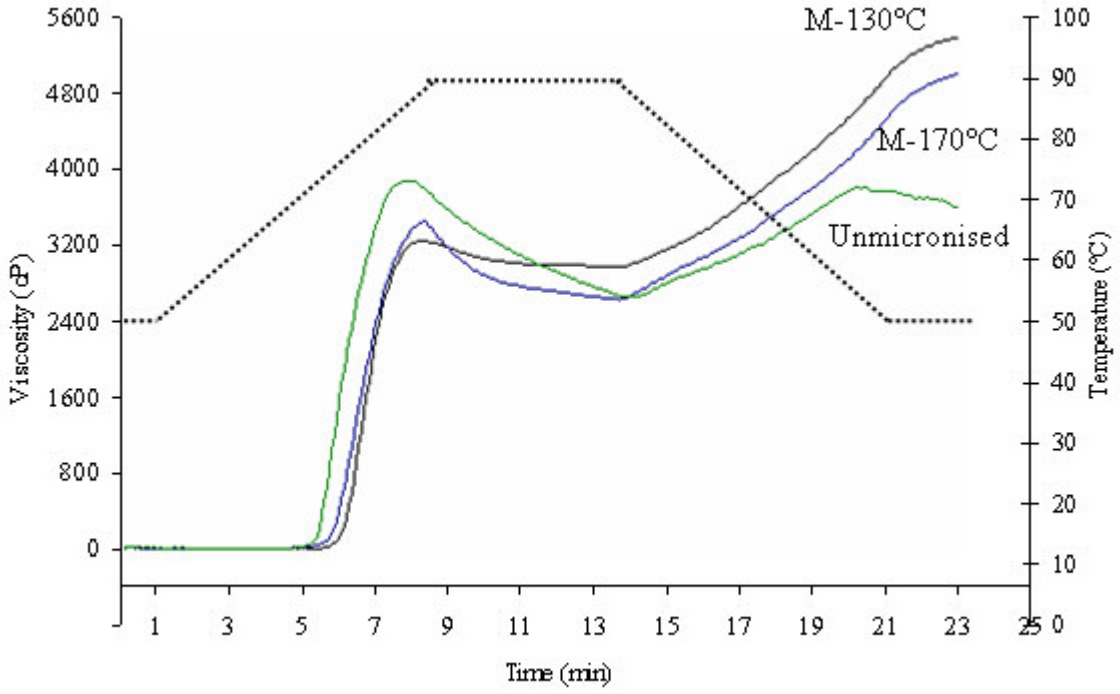

Figure 3.3.9 **Effect of micronisation temperature (130 °C and 170 °C) on pasting properties of cowpea starch isolated from micronised seeds, (Unmicronised= Raw, M= Micronised (41 % moisture, 130 and 170 °C))**

The gelatinised starch in M-170 °C was possibly also depolymerised. Depolymerised polysaccharides cannot reorder to the same extent as relatively intact polysaccharides molecules. Therefore, their contribution towards set back viscosity during the cooling phase would be negligible. In order to ascertain the possible presence of depolymerised starch in the M-170 °C flour, water solubility of starch and its degradation products were analysed. The HPLC-GP results (Figure 3.3.8) do not show extensively depolymerised starch due to micronisation (M-170 °C) i.e., no change in water-soluble sugars and oligosaccharides for this sample. Van den Einde *et al*. (2004) demonstrated that thermal (110, 140, 160 °C) treatment of low moisture (30 and 43 % moisture) corn starch in the absence of shear, resulted in endodegradation of starch and not exodegradation. Exodegradation of starch is indicated by increase in water-soluble products.

The presence of damaged starch, indicative of possible crosslinking and/or depolymerisation, was further elucidated by light microscopy. M-170 °C flour contained intact cells which absorbed acid Fuchsin (Figure 3.3.6 (1c)) and Congo red stains (Figure 3.3.10 (a) and 3.3.10 (b)) indicating the presence of protein and gelatinised starch, respectively.

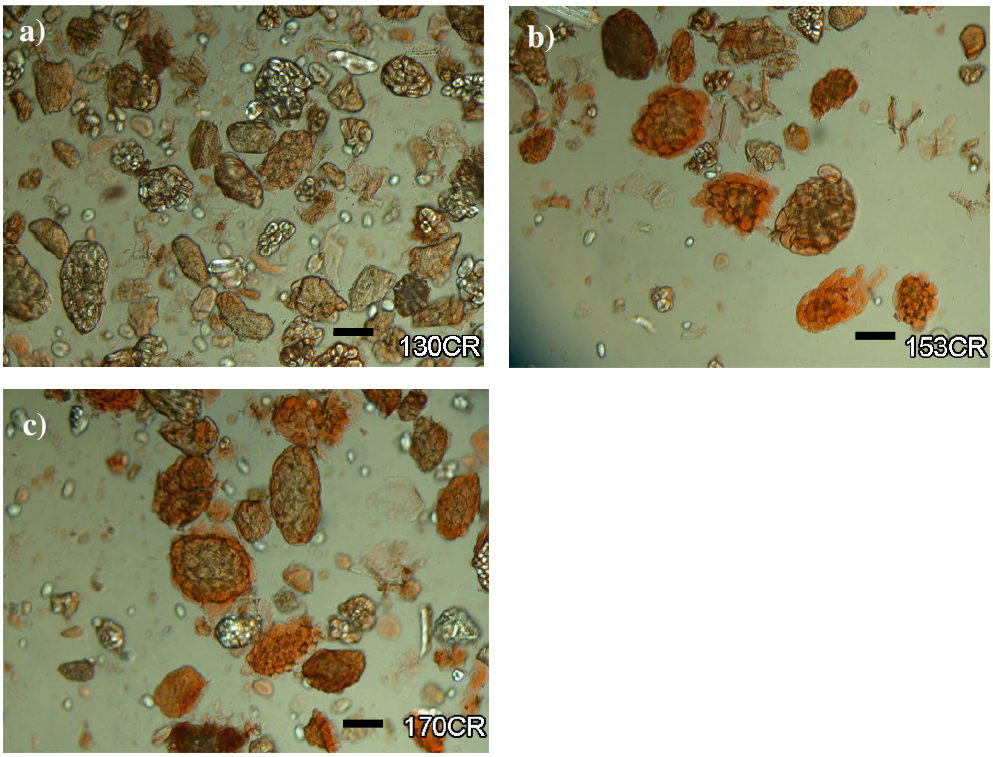

Figure 3.3.10 Effect of micronisation temperatures (130 °C, 153 °C and 170 °C) on cowpea flour from micronised (41 % moisture, infrared heating) seeds stained for damaged starch with Congo red: a = M-130 ºC, b = M-153 °C and c = M-170 ºC, Bar ~ 50 nm

When the cowpeas flour was digested with enzymes, the intact cells were observed to be partially susceptible to α-amylase (Figure 3.3.11 (2b) and 3.3.11 (2c)), and protease (Figure 3.3.11 (3b) and 3.3.11 (3c)) but were indigestible with pectinase ((Figure 3.3.11 (4c) .

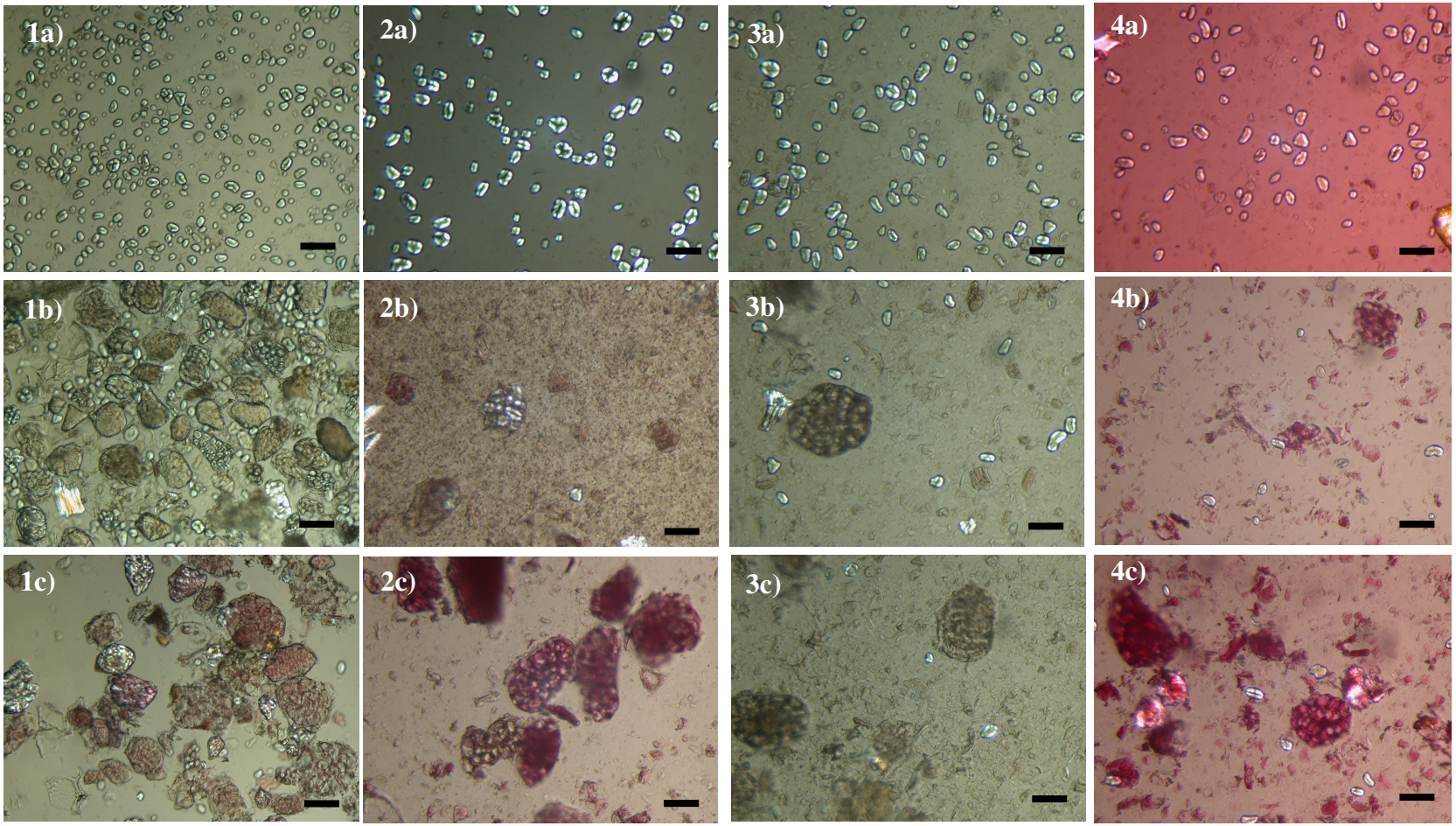

Figure 3.3.11 Effect of micronisation temperatures on cowpea flour from micronised (41 % moisture, 130 °C and 170°C) seeds during sequential enzyme digestion (stained with acid Fuchsin): 1 = No enzyme treatment, 2 = α –amylase, 3 = α –amylase - protease, and 4 = α –amylase-protease- pectin; a = unmicronised, b = M-130 ºC and c = M-170 ºC, Bar ~50 nm

3.3.4 Conclusions

Micronisation of moisture-conditioned cowpeas to temperatures up to 170 °C is beneficial in terms of reduced cooking time of whole seeds. Micronisation of moisture conditioned cowpeas to 130 °C is more effective in reducing the cooking time of cowpeas and would be recommended for this process. This will impact positively on energy and time requirements for cooking dry cowpeas. However, starch functionality is compromised by excessive micronisation as exhibited by M-170 °C cowpea samples.

3.4 Effect of micronisation temperature (130 °C and 170 °C) on functional properties of cowpea flour[4]

Abstract

Functional properties of cowpea flour from moisture-conditioned (41 %) seeds micronised to two different surface temperatures (130 °C and 170 °C) were studied. Micronisation (41 % moisture, 130 °C and 170 °C) significantly ($P \leq 0.05$) increased the water absorption capacity and least gelation concentration of the flour. The treatment significantly ($P \leq 0.05$) reduced the water solubility and swelling indices, gel strength and foaming capacity of the flour. The changes in cowpea flour functional properties, such as the loss of foaming capacity in flours from micronised (41 % moisture, 130 °C and 170 °C) seeds were associated with significant ($P \leq 0.05$) increase in surface hydrophobicity and cross linking of the cowpea protein. SDS-PAGE of the protein-rich fractions revealed changes in the protein subunit profile, which included the formation of disulphide bonds and possibly Maillard reaction derived cross links. The flour from M-170 °C seeds was significantly ($P \leq 0.05$) darker than the flour from unmicronised and M-130 °C seeds.

Keywords: cowpea flour, micronisation, protein, functional properties

[4] Submitted for publication in *Food Chemistry*

3.4.1 Introduction

Micronisation is a short-time, high-temperature process that utilises electromagnetic radiation in the infra red region (wavelength of 1.8-3.4 nm) to rapidly heat materials (Zheng *et al*., 1998). The process has been shown to considerably reduce the cooking time of legumes such as split peas, lentils and cowpeas, (Cenkowski & Sosulski, 1998; Arntfield *et al*., 2001; Phadi, 2004). However, wider use of the micronisation process in cowpeas could be attained by extending the utilisation of micronised legume seeds in the diet beyond the whole seed. Milling of moisture-conditioned and micronised cowpeas into flour could be one such process since there are existing uses of cowpea flour in food systems. There is a wide variety of products that are made from cowpea flour in different parts of Africa which are dependent on its functionality (Phillips *et al*., 2003; Hallén *et al*., 2004; McWatters *et al*., 2005). Apart from the traditional products, cowpea flour has also been used as a nutritious ingredient in fried (Kerr *et al*., 2001) and baked (Phillips *et al*., 2003) products as well as comminuted meat products such as chicken nuggets (Prinyawiwatkul *et al*., 1997b) and meatballs (Serdaroglu *et al*., 2005).

The suitability of cowpea flour in such food systems is dependent on its functional properties such as foaming, water and oil absorption capacities (WAC and OAC) as well as thermally induced gelling (Prinyawiwatkul *et al*., 1997a; Abu *et al*., 2005). Cowpea protein (24 %) is one of the main contributing components to the functionality of the flour. Cowpea seeds have high protein content which is relatively hydrophilic and water soluble (Mwasaru *et al*., 1999a). These physicochemical properties of cowpea protein are crucial in retaining the good foaming properties of the flour which are necessary in imparting a spongy texture of cowpea flour based products such as *akara* (Plahar *et al*., 2006). The WAC and OAC and gelling properties of heterogeneous systems such as flour are a factor of the physicochemical characteristics of protein and starch components (Prinyawiwatkul *et al*., 1997b).

Micronisation of moisture-conditioned seeds has been shown to precook legumes such as peas (Cenkowski & Sosulski, 1998), lentils (Arntfield *et al*., 2001), cowpeas (Phadi, 2004), and beans (Bellido *et al*., 2006). In addition to whole seed products, the flour from

moisture-conditioned and micronised cowpeas has the potential for utilisation in some food systems depending on its functionality. Fasina *et al*. (2001) reported increased pasting viscosities of legume (kidney, pinto, and black beans, and green peas) flour following micronisation (< 10 % moisture, 140 °C). Similarly, Cenkowski and Sosulski (1998) reported significant increase in pasting properties for flour milled from micronised (26 % moisture, 120 °C) split peas. Contrary to these findings, results reported in section 3.3.3.2 show that micronisation of moisture-conditioned (41 %) cowpea seeds, especially at 170 °C adversely reduced the pasting properties of cowpea flour. Fasina *et al*. (1999) reported improved water absorption capacity for hulless and pearled burley following micronisation (26.5 % moisture, 105 and 115 °C). At the same time, a reduction in WAC has been reported for chick pea flour milled from micronised (17 % moisture, 69, 88 and 90 °C) seeds (Sarantinos & Black, 1996). Micronisation (17 % moisture, 69, 88 and 90 °C) has been reported not to have a definite effect on oil absorption capacity of chick pea flour (Sarantinos & Black, 1996). Since information on functional properties of flour from moisture-conditioned and micronised legumes is rather scanty, the objective of this study was to examine the effect of micronising moisture-conditioned cowpea seeds to low (130 °C) and high (170 °C) final surface temperatures on functional properties of the resultant flour.

3.4.2 Material and methods

3.4.2.1 Raw materials

Bechuana white (cream colour) cowpeas supplied by Agricol (Potchefstroom, South Africa) were cleaned to remove chaff, shrivelled and broken seeds. The cleaned seeds were packed in polypropylene bags and stored at 4 °C until the time of use.

3.4.2.2 Hydrothermal process and cowpea flour preparation

The cowpeas were micronised according to the method described in section 3.3.2.2.

3.4.2.3 Colour values of the cowpea flour

Colour of the unmicronised and micronised (41 % moisture, 130 and 170 °C) cowpea flour was determined using a Chroma Meter CR-400 (Konica Minolta Sensing Inc,

Osaka, Japan). The colour of the flours was expressed as L*, a*, b* values, where L*=lightness, a* =redness, and b*=yellowness.

3.4.2.4 Determination of moisture content

Moisture content of the micronised (41 % moisture, 130 and 170 °C) and unmicronised flours was determined using the AACC method 925.10 (American Association of Cereal Chemistry, 2000).

3.4.2.5 Determination of crude protein

Crude protein content in the cowpeas was determined using the Dumas method as reported in section 3.1.2.2.

3.4.2.6 Determination of nitrogen solubility index

Nitrogen solubility index of the flour was determined according to the AACC method 46 - 23 (AACC, 2000). One gram flour samples were dispersed in 50 ml of 0.1M NaCl solution and stirred continuously for 1 hr with the pH maintained at 7. About 20 ml of the suspension was centrifuged (10 000 *g*, 15 min, 4 °C) and the supernatant filtered through a Whatman No 1 filter paper. The nitrogen content of the filtrate was determined using a Leco nitrogen analyser 528 (Leco Africa Pty, Kempton Park, South Africa). Protein content of the filtrate was calculated using 6.25 as the conversion factor. Nitrogen solubility index was expressed as the percentage of sample protein on dry basis.

3.4.2.7 Determination of water solubility index (WSI), water and oil absorption capacities (WAC, OAC)

Water and oil absorption capacity (WAC, OAC) of the flour from unmicronised and micronised (41 % moisture, 130 °C and 170 °C) cowpeas were determined according to the AACC method 56-20 (AACC, 2000) with slight modifications. A 2 g (M0) flour sample was dispersed in 40 ml deionised water or refined sunflower oil and vortexed for 10 min. The samples were centrifuged (1000 *g*, 15 min at 20 °C) and the supernatant decanted. The centrifuge tubes were then inverted for 5 min on a paper towel followed by weighing of the residue (M2). The residue from the water absorption samples was

dried in a hot air oven for 24 h at 50 °C and weighed (M1). WAC, OAC and WSI were calculated as follows:

WAC or OAC = {(M2 –M0)/M0} x 100; and WSI = {(M0-M1)/MO} x 100

Where M0 = Sample weight (db); M1 = Weight of dried residue; M2 = Weight of wet or oily residue.

3.4.2.8 Determination of swelling index

Samples of flour from unmicronised and micronised (41 % moisture, 130 °C and 170 °C) cowpeas were dispersed in deionised water (1:20, w/v) and vortexed for 1 min followed by heating in a water bath at 90 °C for 30 min with intermittent mixing. The heated samples were cooled for 30 s under running water and for 10 min in an ice bath to accelerate gel formation. The tubes containing the gels were centrifuged (4500 *x g*, 20 °C) for 10 min, after which the samples were allowed to stand for 5 min at 24 °C. The supernatant was decanted and the residue weighed. Swelling index was calculated as the ratio of the weight of the final residue to the initial sample weight (Abu *et al*., 2005).

3.4.2.9 Determination of cowpea flour concentration on gelation

Cowpea flour dispersions in deionised water with concentration from 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 % (w/v) were prepared in test tubes. The dispersions were heated in a water bath at 80 °C for 1 h followed by rapid cooling under running cold water. The test tubes were left to set at 4 °C for 2 h. Least gelation concentration was determined as the concentration when sample from the inverted tube did not fall or slip (Adebowale, Olu-Owolabi, Olawumi & Lawal, 2005).

3.4.2.10 Determination of gel strength

The strength of gels formed by the flour from unmicronised and micronised (41 % moisture, 130 °C and 170 °C) cowpeas was determined following the method reported by Abu *et al*. (2005). Eighteen percent (w/v) flour dispersions were heated in a water bath (90 °C) for 50 min. The hot pastes were cooled in running water and allowed to set for 15 min in an ice bath following which the gels with a height of 20 mm were kept at 4 °C for 16 h. Gel strength was measured using a TA-XT2 texture analyser (Stable micro

systems, Goldalming, Surrey, UK). A 20 mm diameter probe (P20) with a punch area of 314.16 mm^2 was used. The probe operated at a pre-test speed of 1 mm s^{-1} and penetrated the gel at the speed of 0.5mm s^{-1}. Force required for the probe to penetrate the gel to 8 mm was recorded as a measure of gel strength.

3.4.2.11 Determination of foaming capacity of cowpea flour

The foaming capacity of the flour from unmicronised and micronised (41 % moisture, 130 °C and 170 °C) cowpeas was determined according to the method reported by Akubor, Isolokwu, Ugbane & Onimawo (2000). A 5 % (w/v) dispersion of the flour in deionised water was whipped using a Power Five mixer (Kenwood Ltd, Hants, England) for 8 min at 24 °C. The foam was quantitatively transferred into a graduated cylinder. Foam volume was expressed as a percentage of the volume occupied by the sample prior to whipping.

3.4.2.12 Extraction of a protein-rich fraction

Cowpea protein was extracted from unmicronised and micronised (41 % moisture, 130 °C and 170 °C) seeds according to a modified method of Mwasaru *et al*. (1999a) and Horax *et al*. (2004a). The flour was defatted with hexane (Flour: Hexane, 1:6, w/v) at 25 °C for 4 h and air dried in a fume hood overnight. Ten percent of the defatted flour in 0.1 M NaOH at pH 8.5 was homogenised using a Ultra Turrax T25 (Janke and Kunkel GmbH & Co., K.G., Stauffen, Germany) at 24000 rpm for 30 min (4 °C) followed by centrifugation at 10 000 *g* (4 °C) for 1 h. The residues were extracted twice in 0.1 M NaOH at pH 8.5. The supernatants were pooled and precipitated by adjusting the pH to 4.5 using 0.1 M HCl. The precipitated protein was recovered by centrifuging followed by three washings with deionised water (pH 4.5). The protein isolate was solubilised by adjusting the pH to 7.0 followed by 24 h dialysis (pore size of 12-14 kDa, Labretoria, Pretoria, South Africa) at 4 °C. The dialysed material was freeze dried and termed protein-rich fraction (PRF). The nitrogen content of the PRF was determined using a Leco Nitrogen Analyzer 528 (Leco Africa Pty, Kempton Park, South Africa). Protein content of the PRF was calculated using 6.25 as the conversion factor.

3.4.2.13 Determination of protein surface hydrophobicity

Surface hydrophobicity of the PRF samples was determined according to the method of Hayakawa and Nakai (1985). Ten millilitre solutions of protein were made in 0.01 M phosphate buffer (pH 7) with concentrations ranging from 0.0001 to 0.0008 % (w/v). The probe for aromatic hydrophobicity, 1-anilino-8- naphthalene sulphonate (ANS) (25 µl, 8 mM in 0.01 M phosphate buffer (pH 7.0)) was added into each protein solution, and fluorescence intensities of these solutions were measured at 390 nm excitation and 470 nm emission using an LS 30 Luminescence spectrometer (PerkinElmer Inc, Boston, MA). The surface hydrophobicities, expressed as a slope of fluorescence intensity (arbitrary units) against protein concentration were calculated by linear regression (Statistica 6.0).

3.4.2.14 Determination of dityrosine

Formation of dityrosine in moisture-conditioned and micronised samples was determined according to the method of Davies, Delsignore & Lin (1987). Protein-rich fraction was dissolved in 0.1 M HEPES buffer (pH 7.0) to make a 0.53 mg protein in 2 ml solution. Dityrosine fluorescence was measured at 325 nm excitation and 410 – 420 nm emission using an LS 30 Luminescence spectrometer (PerkinElmer Inc, Boston, MA).

3.4.2.15 Gradient SDS –PAGE of the protein-rich fraction (PRF)

The effect of micronisation temperature on the molecular size and distribution of cowpea proteins was studied using gradient SDS-Gel electrophoresis according to the method reported by Byaruhanga, Erasmus and Taylor (2005). The gels (12.5 x 16 cm, 1.5 mm thick) had a concentration gradient from 4 to 18 % and were polymerised with 0.1 % (w/v) ammonium persulphate (APS) and tetramethylenediamine (TEMED). The protein was dispersed in sample buffer (0.125 % (w/v) Tris/HCl, 20 % (v/v) glycerol, 2 % (w/v) SDS and 0.005 % (w/v) bromophenol blue). For the electrophoresis under reducing conditions, 0.1 % 2-mercaptoethanol was added to the sample buffer. The gels were loaded to a constant protein content of 49.5 µg. Molecular weight marker solution, low range (Roche Diagnostics Corporation, Indianapolis, USA) was diluted by 1 to 10 with reducing sample buffer. The mixture consisted of phosphorylase B (M_r 97.4 x 10^3),

bovine serum albumin (M_r 66.2 x 10^3), aldolase (M_r 39.2 x 10^3), triose phosphate isomerase (M_r 26.6 x 10^3), trypsin inhibitor (M_r 21.5 x 10^3) and lysozyme (M_r 14.4 x 10^3). The diluted molecular weight marker was boiled for 5 min and 20 µl (96 µg protein molecular weight marker) was loaded on the gels. Electrophoresis was carried out at a constant voltage of 25 mA per gel and 150 V for 14 h at 8 °C using a Protean II xi vertical cell with a 1000 Powerpac (Bio-Rad Laboratories, Hercules, CA). Proteins were stained with 0.03 % (w/v) Coomassie Brilliant Blue R250 in 7 % (v/v) acetic acid and 20 % (v/v) methanol and 3.2 % trichloroacetic acid (TCA). After staining the gels were de-stained with 4 % (v/v) acetic acid and 29 % (v/v) methanol and 3 % TCA. The de-stained gels were scanned on a flat bed scanner.

3.4.2.16 Statistical analysis

Mean values for the functional properties, colour and surface hydrophobicity of the cowpea flours were obtained from three repetitions. One way analysis of variance (ANOVA) of the data and correlations of variables was performed using Statistica version 6 (StatSoft, Inc., Tulsa, OK) statistical software. The least significance difference test at $P \leq 0.05$ was used to separate the means.

3.4.3 Results and discussion

Cowpea flour from unmicronised seeds contained 23.9 % crude protein which is within the range reported for cowpeas (Chan & Phillips, 1994). Although there were no prominent changes in crude protein content with micronisation (41 % moisture, 130 and 170 °C) (Table 3.4.1), the amount of protein extracted as well as the purity of the PRF declined with increasing micronisation temperature (Figure 3.4.1). The protein recovery attained in this work is higher than what some workers have obtained for cowpea, although the protein content of the PRF is within the range reported for cowpea protein isolates (Mwasaru *et al*., 1999a)

Micronisation (41 % moisture, 130 °C and 170 °C) significantly ($P \leq 0.05$) reduced the lightness of cowpea flour with increasing temperature (Table 3.4.1). Cowpea flour from the M-170 °C seeds was the darkest of the samples and it had higher yellowness and

redness values. The browning of the cowpea flour with micronisation was possibly due to Maillard reactions since cowpeas do contain reducing sugars (Longe, 1980; Phillips *et al*., 2003) and have high protein content.

Table 3.4.1 Effect of high (170 °C) and low (130 °C) final micronisation temperature for cowpea seeds (41 % moisture) on physicochemical properties of cowpea flour

Physicochemical property	Micronisation		
	[y]Unmicronised	130 °C	170 °C
Moisture	9.2[a] (0.7)	5.0[b] (0.9)	4.8[b] (0.5)
Protein	24.2[c] (0.2)	24.7[b] (0.3)	25.9[a] (0.6)
L*	84.36[a] (0.4)	81.34[b] (0.6)	70.06[c] (0.3)
a*	5.00[c] (0.0)	5.23[b] (0.16)	8.45[a] (0.3)
b*	6.01[b] (0.2)	6.28[b] (0.4)	13.33[a] (0.3)
Protein solubility (%)	87.4[a] (13.7)	59.7[b] (13.0)	47.7[c] (5.7)
PRF surface hydrophobicity	228[c] (45)	369[b] (34)	624[a] (23)

Means followed by the same letter within a row are not significantly different at level $P \leq 0.05$; standard deviations of the means are in parenthesis, L*= lightness, a* = redness, b*= yellowness, PRF = Protein-rich fraction, [y] Unmicronised = Raw

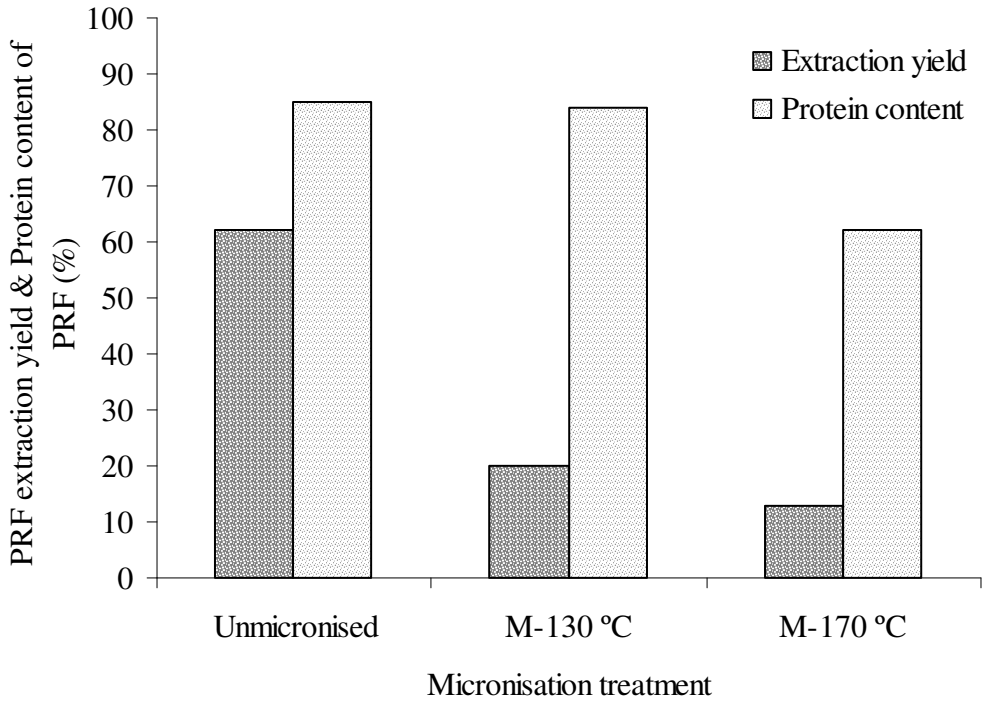

Figure 3.4.1 Effect of high (170 °C) and low (130 °C) final micronisation temperature for cowpea seeds (41 % moisture) on recovery of protein rich fraction (PRF) from cowpea seeds and its protein content (purity); (Yield has been expressed as percentage of crude protein content of the cowpeas)

Protein solubility in an aqueous environment is an essential property that affects functionality of cowpea flour in terms of foaming, emulsification and gelation (Nnanna, Phillips, McWatters & Hung, 1990). Nitrogen solubility index of unmicronised flour was comparable to values reported in literature (Abu *et al.*, 2005) (Table 3.4.1). Micronisation (41 % moisture, 130 °C and 170 °C) reduced ($P \leq 0.05$) the NSI of the cowpea flour by 32 and 45 %, for the M-130 °C and M-170 °C flours, respectively.

Micronisation (41 % moisture, 130 °C and 170 °C) caused the denaturation of the cowpea protein hence reducing its solubility. Similar reductions in protein solubility have been reported in micronised peas (Cenkowski & Sosulski, 1998), lentils (Arntfield *et al.*, 1997;

Arntfield *et al*., 2001) and beans (Bellido *et al*., 2006). Zheng *et al*. (1998) attributed the reduction in protein solubility for micronised (18 % moisture, 140 °C) legumes to hydrophobic interactions which render the protein less soluble in water. Cowpea protein is generally hydrophilic (Horax *et al*., 2004a) indicating that most of the non polar/hydrophobic side chains are buried inside the protein. The protein-rich fraction from unmicronised cowpeas had lower surface hydrophobicity values as compared to what is reported for cowpea protein isolate (Table 3.4.1) (Horax *et al*., 2004a). The difference in surface hydrophobicity of the cowpea proteins could be due to difference in variety (Horax *et al*., 2004a) and protein isolation methods. However, micronisation (41 % moisture, 130 °C and 170 °C) significantly ($P \leq 0.05$) increased the surface hydrophobicity of the PRF possibly by changing the cowpea protein conformation to expose more hydrophobic sites. The PRF from the M-170 °C had significantly higher surface hydrophobicity than the PRF from M-130 °C cowpeas (Table 3.4.1). This may imply that more hydrophobic sites were exposed with increasing micronisation temperature.

Furthermore, possible complexion of phenolic compounds and tannins with protein could contribute to the reduction in protein solubility of the micronised (41 % moisture, 130 °C and 170 °C) cowpeas (Chang, Collins, Bailey & Coffey 1994). Phenolic compounds and tannins have been reported in cowpea varieties (Chang *et al*., 1994; Cai, Hettiarachchy & Jalaluddin, 2003). Phenolic compounds are oxidised to quinones that react irreversibly with sulphydryl and amino groups of proteins hence reducing protein solubility (Damodaran, 1996a).

Cowpea flour from unmicronised seeds absorbed 1137 g kg^{-1} oil and micronisation (41 % moisture, 130 °C and 170 °C) did not change this attribute (Table 3.4.2). Prinyawiwatkul *et al*. (1997a) reported that oil absorption capacity of cowpea flour does not change with most processing treatments such as boiling, milling particle size and fermentation. The presence of non-polar side chains, which bind the hydrocarbon side chain of oil would promote oil binding capacity of flours, however in this study the significantly ($P \leq 0.05$) higher surface hydrophobicity (Table 3.4.1) of the protein-rich fraction isolated from

cowpeas (41 % moisture) micronised to 170 °C did not enhance the OAC of the flour. Oil absorption capacity is an important property for cowpea flour that would be used as an extender in comminuted meat formulations such as meat balls and sausages where flavour retention and palatable mouth feel is desired. Since micronisation (41 % moisture, 130 °C and 170 °C) did not change the OAC of the flour, flour from moisture-conditioned and micronised seeds could be used to extend meat products without adverse effect on texture and mouth feel (Prinyawiwatkul *et al*., 1997b; Serdaroglu *et al*., 2005).

Table 3.4.2 Effect of high (170 °C) and low (130 °C) final micronisation temperature for cowpea seeds (41 % moisture) on functional properties of cowpea flour

Functional property	Micronisation		
	[y]Unmicronised	130 °C	170 °C
Oil absorption capacity (g kg^{-1})	1137 (174)	1208 (177)	1177 (147)
Water absorption capacity (g kg^{-1})	1384[c] (89)	2509[b] (75)	2871 [a] (101)
Water solubility index	38.6[a] (1.6)	22.6[b] (1.51)	17.4 [c] (1.6)
Swelling index	7.24[a] (0.43)	5.95[b] (0.44)	5.92 [b] (0.39)
Gel strength (Nmm^2)	115.7[a] (8.9)	72.3[b] (4.6)	28.8 [c] (2.5)
Least gelation capacity (w/v)	8[c] (0.8)	11[b] (1.0)	13[a] (1.0)
Foam capacity (%)	291[a] (11.5)	112[b] (2.1)	102 [c] (2.1)

Means followed by the same letter within a row are not significantly different at level $P \leq 0.05$; standard deviations of the means are in parenthesis [y] = Raw

Micronisation of moisture-conditioned cowpea seeds (especially 170 °C) significantly ($P \leq 0.05$) increased the WAC of the cowpea flour (Table 3.4.2). The flour from M-170 °C cowpeas absorbed more ($P \leq 0.05$) water than M-130 °C samples. Fasina *et al*. (1999) reported that micronisation (26 % moisture, and 115 °C) improved the water holding capacity of hulless and pearled barley flour. Prinyawiwatkul *et al*. (1997a) also

reported improved water retention capacity for cowpea flour from soaked and boiled seeds. Starch and protein are important constituents that determine water absorption properties of heterogeneous systems such as flour. Fasina *et al*. (2001) demonstrated that micronisation (<10 % moisture, 140 °C) of legumes (pinto and black beans, lentils and green peas) resulted in increased WAC which was mainly attributed to protein denaturation since the amount of starch gelatinised under the micronisation conditions was minimal.

Despite the increase in water absorption capacity with micronisation (41 % moisture, 130 °C and 170 °C), swelling index of the flour had a significant ($P \leq 0.05$) negative correlation ($r = -0.83$) with WAC. Micronisation (41 % moisture, 130 °C and 170 °C) significantly ($P \leq 0.05$) reduced the swelling index of the flours by 17.8 and 18.2 % for the M-130 °C and M-170 °C, respectively (Table 3.4.2). Swelling index is indicative of starch granule swelling during gelatinisation as well as water retention due to protein gelation. It has been reported that the starch in moisture-conditioned and micronised legumes is gelatinised and protein solubility is reduced (Arntfield *et al*., 1997; Bellido *et al*., 2006). Hence the flour from micronised (41 % moisture, 130 °C and 170 °C) cowpeas had lower swelling indices than the unmicronised cowpea flour. Similar reduction in swelling index has been reported in irradiated cowpea flour (Abu *et al*., 2005).

Cowpea flour from unmicronised seeds had a water solubility index (WSI) of 38.67 which was significantly ($P \leq 0.05$) reduced by 42 and 55 % by micronisation to 130 °C and 170 °C, respectively (Table 3.4.2). Water solubility index is an indication of the water soluble fractions in the flour such as protein and sugars. WSI had a significant positive correlation with NSI of the flour. Hence the reduced WSI could partly be due to the reduced protein solubility.

Cowpea flour from unmicronised cowpeas had the lowest ($P \leq 0.05$) gelation concentration of 8 % and formed a significantly ($P \leq 0.05$) stronger gel at 18 % concentration than the flour samples from moisture-conditioned seeds micronised to

130 °C and 170 °C cowpeas (Table 3.4.2). The least gelation concentration found in this work is within the values reported for cowpea flour (Olaofe *et al*., 1993; Prinyawiwatkul *et al*., 1997a). Micronisation (41 % moisture, 130 °C and 170 °C) increased the lowest gelation concentration to 10 and 12 % for the flour from moisture-conditioned cowpeas micronised to 130 °C and 170 °C, respectively. The strength of the gels formed from the 18 % (w/v) dispersion was significantly ($P \leq 0.05$) reduced by 39 and 75 % for the M-130 °C and M-170 °C flours, (Table 3.4.2). Prinyawiwatkul *et al*. (1997a) reported similar increase in the least gelation concentration for cowpea flour following soaking and boiling. In a heterogeneous system where both the protein and starch are in their native form, both protein and starch contribute towards the gelation properties of the flour. As indicated in section 3.3.3.2, micronisation of moisture-conditioned cowpea seeds to 170 °C severely reduced the pasting viscosities of the milled flour than the mild 130 °C micronisation treatment. Consequently the significant ($P \leq 0.05$) increase in the least gelation concentration and the weak gel formed by the cowpea flour from seeds micronised to 170 °C was most likely due more to protein gelation than starch gelation. In addition, Prinyawiwatkul *et al*. (1997a) reported that high protein (denatured) flour with pregelatinised starch require greater flour concentration for thermal gel formation to occur.

Foaming reflects the capacity of protein to form stable layers surrounding gas droplets in a liquid phase. In order for this to be possible, the proteins need to be soluble in the aqueous phase and be in a position to diffuse and concentrate at the air/water interface and partially unfold to form a cohesive layer around the gas bubbles as well as possess sufficient viscosity and mechanical strength to prevent rupture and coalescence of droplets (Damodaran, 1996a). Cowpea flour from unmicronised seeds formed 191 ml of foam as compared to the 12 and 2 ml formed by M-130 °C and M-170 °C flour from the moisture-conditioned cowpeas micronised to 130 and 170 °C, respectively (Table 3.4.2). Similar reduction in foaming capacity has been reported in irradiated cowpea flour (Abu *et al*., 2005). The significant ($P \leq 0.05$) reduction in foaming capacity of micronised (41 % moisture, 130 °C and 170 °C) samples was possibly due to extensive protein denaturation. Foaming capacity had a significant ($P \leq 0.05$) positive correlation ($r = 0.79$)

with protein solubility index of the flour. Aluko and Yada (1993) reported that protein solubility has a positive correlation with foaming capacity/expansion while aliphatic hydrophobicity has a negative impact. In addition, Townsend and Nakai (1983) reported that protein flexibility is a crucial property for protein stabilised foams. Reduction in foaming properties of partially purified cowpea globulin treated with microbial calcium-independent transglutaminase has shown that protein crosslinking may lead to gradual loss of flexibility and the proteins' ability to unfold at the water air interface (Aluko & Yada, 1999).

SDS-PAGE of the extracted protein-rich fraction from the unmicronised cowpeas showed two main (56 and 50 kDa) and two minor (63 and 61 kDa) bands in the region between 39.2 and 66 kDa and 5 minor bands in the 20 to 39 kDa regions (Figure 3.4.2 (a)). These bands correspond to the four major polypeptides (65, 60, 56 and 50 kDa) reported by Chan and Phillips (1994) in the globulin fraction. The cowpea albumins have bands in the 90 - 100 kDa regions and minor bands were observed in this region (Figure 3.4.2 (a)). Rangel *et al*. (2003) observed two major bands 50 and 52 kDa following SDS-PAGE of cowpea protein isolate. These are in the typical molecular mass range for 7S storage proteins (Horax *et al*., 2004a).

Although disulphide links have been reported in the α- vignin component of purified cowpea globulins involving a 60 and a 20 subunits, there was no distinct changes in the subunit profile of the PRF from unmicronised cowpea seeds under reducing conditions (Figure 3.4.2 (b)). There was relative reduction in the size of the 2 major bands in the protein isolated from moisture-conditioned cowpea seeds micronised to 170 °C (Figure 3.4.2(a)). The reduction in size could possibly be due to associations involving the 52 and 55 kDa polypeptides in the formation of higher molecular weight polymers that could not pass through the gradient gels.

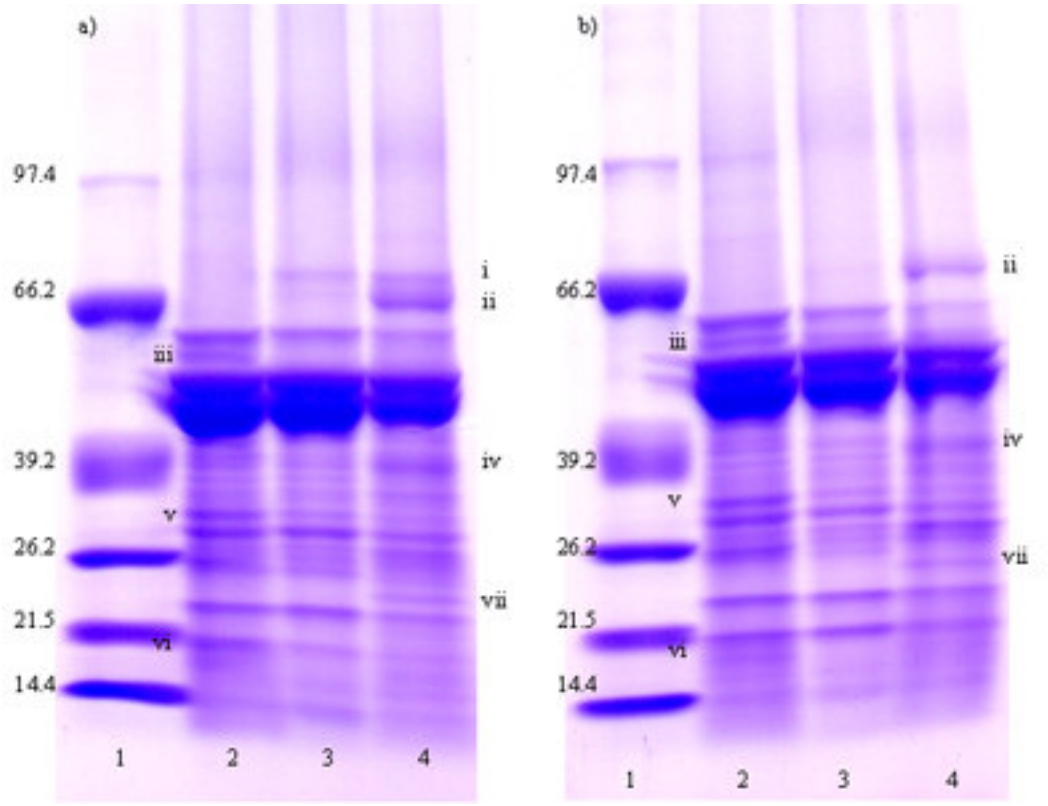

Figure 3.4.2 SDS-gradient gel electrophoresis profiles of cowpea protein-rich fraction from unmicronised (raw) and micronised (41 % moisture, 130 and 170 °C) seeds under non reducing (a) and reducing conditions (b): lane 1 = molecular markers; lane 2 = unmicronised; lane 3 = micronised to 130 °C and lane 4 = micronised to 170 °C (changes in protein band profile are denoted with (i) to (vi))

Micronisation (41 % moisture, 130 °C and 170 °C) also resulted in the formation of 2 minor bands, at 69 and 73 kDa (Figure 3.4.2(a – i) and 3.4.2 (a-ii)), and the 69 kDa (Figure 3.4.2(a –ii)) band was prominent in the fraction extracted from moisture-conditioned cowpeas micronised to 170 °C. The 73 kDa (Figure 3.4.2(a-i)) was possibly a result of disulphide cross-links involving the 21 kDa and 61 kDa monomers, since it was reduced by mercaptoethanol (MCE) (Fig 2 (b)). The 61 (Figure 3.4.2(a) and 3.4.2 (b-iii)) and 21 kDa monomers (Figure 3.4.2(a) and 3.4.2(b-vi)) disappeared in micronised

(41 % moisture, 130 °C and 170 °C) samples under non-reducing conditions and reappeared under reducing conditions (Figure 3.4.2(a) and 3.4.2(b)). Freitas *et al*. (2004) reported that the γ – Vignin (22 kDa) fraction of cowpea globulins have an intra-polypeptide disulphide bond. It is possible that micronisation of moisture conditioned seeds especially to 170 °C resulted in the reformation of these bonds leading to the formation of interpolypeptide disulphide cross linking.

In addition to disulphide cross linking, it is evident that other forms of cross linking may have taken place during micronisation (41 % moisture, 130 °C and 170 °C). Other bands that were present (Figure 3.4.2(a); 3.4.2(b – ii); 3.4.2(b-iv); 3.4.2(b- vii)) in the protein-rich fraction from moisture-conditioned cowpeas micronised to 170 °C were not reduced by MCE and could possibly be due to other forms of cross-linking that involved the 2 prominent bands and band (v) (Figure 3.4.2 (b-v)), since these bands decreased with micronisation temperatures both under reducing and non reducing conditions (Figure 3.4.2(a) and 3.4.2(b)). Maillard reactions might have led to the formation of some crosslinks in the cowpeas protein (Gerrard, 2002). The decrease in L* values with increasing micronisation temperatures indicate that Maillard-type browning reactions possibly occurred in micronised (especially the 170 °C) sample. The higher end micronisation temperature (170 °C) resulted in darker flour than the lower micronisation temperature (130 °C) (Table 1). Cowpeas contain both reducing and non reducing sugars (Longe, 1980; Phillips *et al*., 2003) which could result in both Maillard and caramelisation reactions. In addition, dityrosyl and isopeptide cross links would also be another possible form of crosslinking (Singh, 1991; Gerrard, 2002) due to the severe heat treatment especially for the flour from the moisture-conditioned cowpeas micronised to 170 °C. Measurement of dityrosine formation was not successful due to the presence of impurities in the extracted protein-rich fractions.

3.4.4 Conclusions

Micronisation of moisture-conditioned cowpeas severely affects the functionality of cowpea protein resulting in the loss of foaming capacity and reduction in gelation capacity, solubility index and swelling. Despite this reduction, mild micronisation

temperatures can still be used to process flour with modified functionality. However, flours from moisture-conditioned cowpeas micronised to high temperatures have limited application in food systems due to the decline in most of the functional properties measured in this study.