CHAPTER 3: DEVELOPMENT OF A NON-DESTRUCTIVE IMAGE ANALYSIS (IA) TECHNIQUE FOR THE QUANTITATIVE MEASUREMENT OF MAIZE KERNEL TRANSLUCENCY

3.1 MATERIALS AND METHODS

3.1.1 Selection and preparation of kernels

Intact cobs of five white and three yellow dent maize industrial cultivars, (F2 segregating hybrids), supplied by Syngenta Seed Co (Pty) Ltd. were used for sampling of kernels. Samples were produced under irrigation at Delmas, Mpumalanga province, South Africa during the 2000 - 2001 growing season. The maize was allowed to dry naturally on the plant on the land before harvesting. Only sound intact maize kernels were used for IA and vitreousness measurements. Kernels considered damaged were: discoloured (including yellow kernels on a white cob due to accidental cross-pollination), poorly developed (obvious lack of proper endosperm development compared to the rest of the kernels on the cob), deformed (for example germ positioned on an abnormal place on the kernel), or abnormally small (relative to the other kernels on the cob, or less than 4.0 mm in length) kernels or those showing chips, fractures, stress cracks, or mechanical, insect or fungal damage. Kernels were removed from the cob by hand, then cleaned using a 4.0 mm opening sieve. Kernels were gently rubbed over the sieve by hand to remove excessive small kernels, pieces of bran coming from the cob and any other debris. After cleaning, kernels were stored in dry plastic containers with lids in the cool and dark. Where applicable for large samples, kernels were counted using a seed counter. Damaged kernels were removed from the samples before analysis. The number of kernels removed per 100 kernels varied from 5 to 15. A few of the cobs showed clear signs of fungal damage, leading to a higher proportion of damaged kernels. As the cultivars were dried naturally on the land, the percentage of stress cracks was low and most unsound kernels were removed due to size (too small) or fungal damage.

Table 3.1 Description of the South African maize cultivars used in the experimental work

Description	Allocated code number	Genetic history	Colour
SR 52 Bethal 00/01*	1	F2 cross	White
L390 I/K., 500/01: 434	2	F2 cross	White
CRN 3549 00/01: 429	3	F2 cross	White
R827/I, 500/01: 435	4	F2 cross	White
CRN 3549 500/01: 439	5	F2 cross	White
N282/FO, 500/01: 467	6	F2 cross	Yellow
N290, K, 500/01: 460	7	F2 cross	Yellow
N258 K/I, 500/01: 460	8	F2 cross	Yellow

* "01" referred to the year of harvest (August 2001)

3.1.2 Image Analysis

Kernels were analysed using a Leica Q-Win Q500 IW-DX Image Analyser (Leica Imaging Systems Ltd., Cambridge, United Kingdom) fitted with Leica Q-Win standard Microsoft Windows compatible software (Windows 95 software). The system was also fitted with a Sony XC-75 CCD Camera B/W (Sony, Tokyo, Japan) with 560 lines (fitted with a 35 mm zoom lens), a standard resolution image capture board (600 dpi) and a standard personal computer system and a 17 inch high resolution monitor. The spatial resolution of the images was 764 X 575 pixels and the calibration factor was 0.22 mm/pixel (both for x and y).

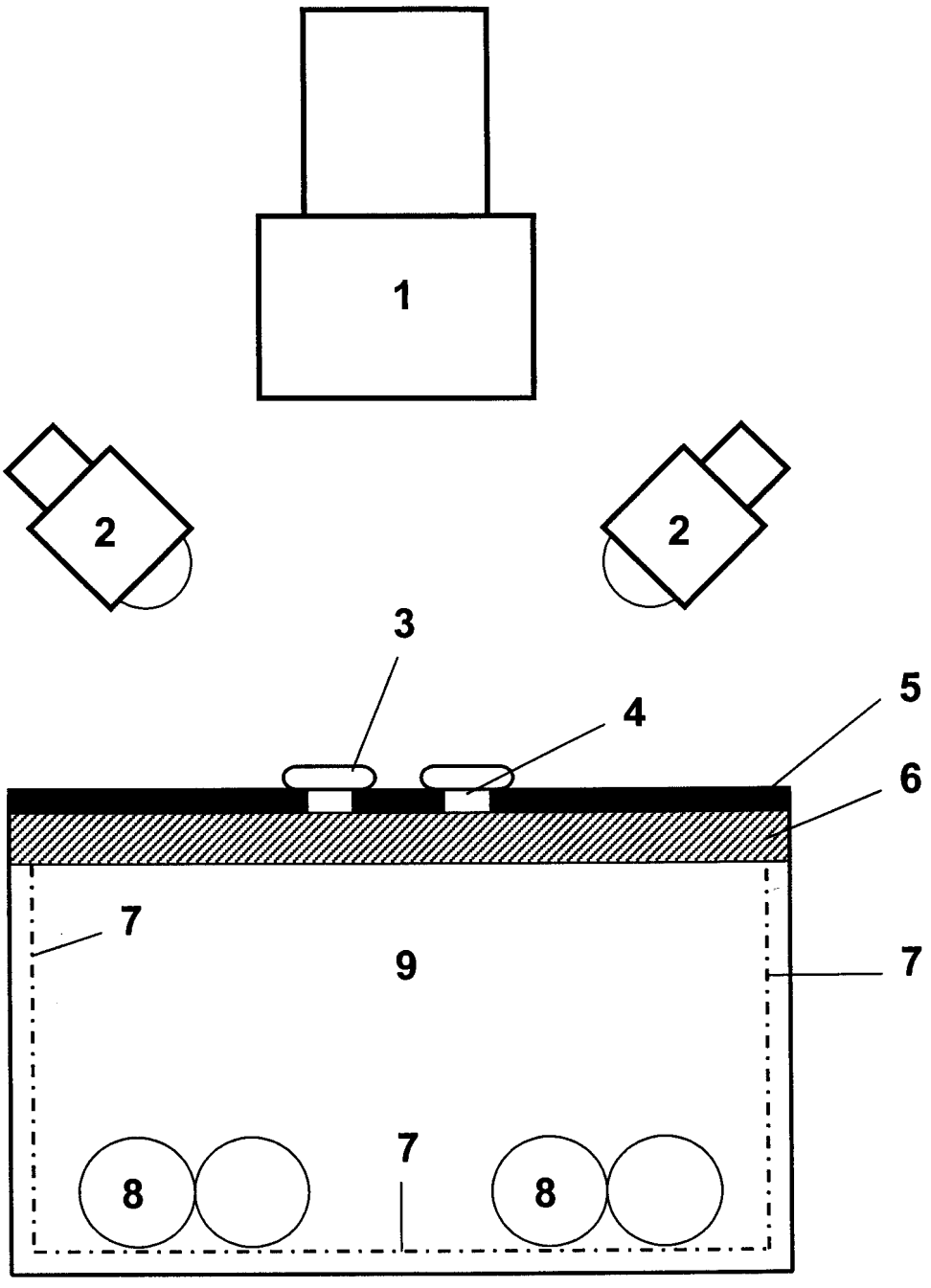

Figure 3.1 General design of maize translucency detection equipment: 1, digital camera with 35 mm lens; 2, incandescent lamps (40 W) for kernel illumination from above; 3, maize kernel positioned on top of circle; 4, round hole in black paper (circle); 5, black paper cover; 6, white Perspex layer; 7, mirrors covering inside walls; 8, double-tubed fluorescent lamps (11 W); 9, light box

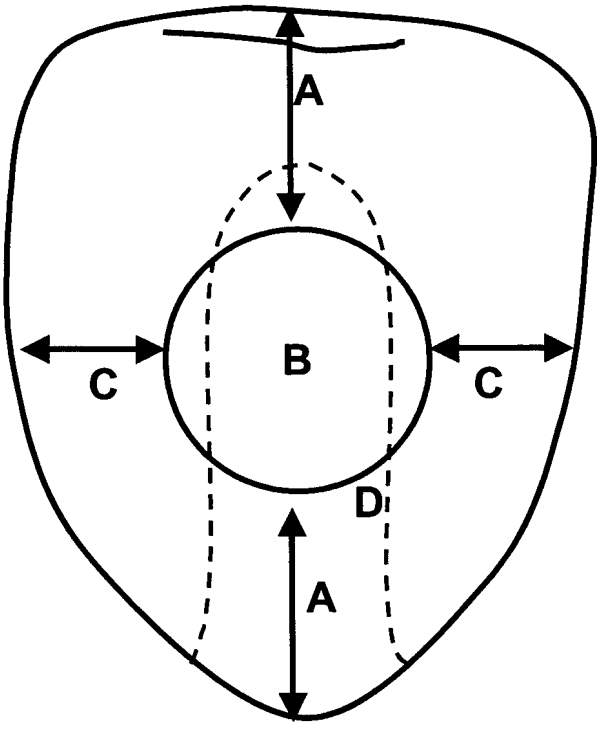

Figure 3.2 The positioning of an intact whole maize kernel on a circle to achieve translucent images. The distances marked "A" were the same and the two distances marked "C" were the same. Area B shows the position of the light circle beneath the maize kernel and line D shows the border of the germ on top of the kernel

Maize kernels were placed on a wooden light box (Fig 3.1), with a length and breadth of 50 cm and a depth of 30 cm. Two double-tubed fluorescent lamps (11 W, Osram Dulux G23 energy savers of 900 Lumen intensity, Lumilux, Italy) were placed next to each other inside the box. The box was fitted with mirrors totally covering all inside surfaces except the top surface. White Perspex (3 mm thick) was placed over the top and covered with black paper with round holes (circles) punched into it to resemble a mask. By placing kernels on top of the circles in the mask (Fig 3.2), partial illumination from below was achieved, instead of full illumination achieved by using modelling clay surrounding individual kernels on the light table (Felker and Paulis 1993). Light produced from uncovered areas was screened off as the circles were smaller than the kernels. Four additional incandescent lamps (40 W soft white, Osram, Italy) for illumination of the kernels from above were also placed

above the unit (Fig 3.1). All work was done in a dark room and a black cloth for additional covering of the camera and light box stand including all light fixtures was used. Three attributes per kernel were measured: 1, The projected kernel area (reflected light image); 2, Projected area of translucent endosperm (gray transmitted light image through the circle mask) and 3, Germ size measurement as determined by hand editing. The three measurements were combined for each kernel for analysis. Seven different sized circles were investigated (Table 3.2).

Table 3.2 Sizes of the circles in the paper mask used as a light source for detecting maize kernel translucency

Circle surface area (mm^2)[a]
17.2 (0.19)
29.6 (0.58)
43.8 (0.70)
48.3 (0.60)
66.2 (0.85)
88.4 (0.91)
113.4 (0.86)

a Means and standard deviation of circles

The IA system was set-up to accommodate a total of 49 (7 x 7) kernels simultaneously. Depending on the lens size, a large number of kernels can be measured simultaneously (100 or more circles are possible on the light box). The camera was set up at a fixed calibration and no changes were made during measurements. The camera was used with the shutter fully open and the Image Analyser video intensity gain/offset signals were adjusted for maximum picture contrast. For this system the gain/offset percentages were 50%/25%. At maximum contrast, the image background (black mask) had a gray value of 0 (black). Image brightness was optimised by ensuring that all gray levels were stretched as much as possible across the spectrum from

black to white (0 – 255). Transmitted light images of each kernel were captured after kernels were illuminated through the circle in the paper mask (Fig 3.2). The gray threshold level was set at a minimum of 54 for white kernels and at 44 for yellow kernels. All images captured were in monochrome (gray). The images were segmented for the gray value range 54 to 255 for white and 44 to 255 for yellow kernels, creating a binary mask image used for the measurement of the total translucent area of the kernels. The minimum threshold levels of 54 for white maize and 44 for yellow maize were chosen because they were sensitive enough to detect gray pixels from the samples with a small percentage of translucent endosperm, while still allowing sufficient scope for detecting pixels from samples with a large percentage of translucent endosperm, with a minimum of overexposure. By using the above levels, 201 levels of gray for white and 211 levels of gray for yellow maize per pixel can be detected allowing for a sufficient range of detection. Binary images were edited after detection using the features available in the Image Analyser software (Leica Imaging Systems Ltd., Cambridge, United Kingdom).

Translucency was measured with the germ facing up towards the camera. This was to allow sufficient light to enter the kernel via the circles without unnecessary scattering caused by the germ, as the germ covers most of the light exposure area. Although moisture content did not influence the readings significantly, excessive moisture ranges were avoided. A kernel moisture range of 10 – 14% (g moisture/100 g kernels) was used.

3.1.3 Effect of humidity exposure on the translucency of intact maize Kernels

As the effect of humidity was a concern with regard to its possible influence on translucency measurements, the translucency of kernels before and after exposure to a high humidity environment was measured. Three intact maize kernels of three cultivars of known moisture content determined by AACC method 44-18 (American Association of Cereal Chemists 2000) were

selected. The kernels were taken from samples stored at ambient conditions (Pretoria Relative Humidity fluctuating between 30 and 60% during the summer). Translucency was measured in triplicate as described in section 3.1.2. The kernels were then placed in a desiccator containing a saturated potassium dichromate solution (relative humidity 98%) (Stokes 1948). The container with the kernels was left at 25°C for 5 days. After exposure to the high humidity level, the translucency of the kernels was measured again.

3.1.4 Effects of translucency measurement methodology

3.1.4.1 Orientation of kernel position in relation to the direction of detection

Three white kernels were used for the assays. Measurements were made in triplicate on the same sized circle (29.6 mm^2). Measurements were done using two kernel orientations, namely vertical with kernel germ and tip cap direction facing 90° and horizontal with kernel germ direction facing 0°. The germ side faced the camera lens.

3.1.4.2 Binary amendment

Although some maize kernels gave a continuous translucent area with detection, most kernels gave two separate translucent areas. This was due to the distribution of opaque endosperm in the South African hybrids. Most of the opaque endosperm is in the middle section of the kernel. Therefore, most cultivars produced an image consisting of two vitreous sections on either side of the opaque middle section. This was not a problem if only one kernel was analysed, but when multiple kernels were analysed simultaneously, it was necessary to combine the area measurements of the two sides of each kernel by using the available "amendment" software.

Three types of amendments were tested to combine the area measurements for each kernel. A set of three kernels was used. Kernels, circle size, gray thresholds and orientation were kept the same.

Binary amendments tested were (Leica QWin User Guide 1996):

- Amendment one - the area measurements of each separate area combined manually.
- Amendment two - the combined area measurements using the vector element.
- Amendment three - the combined area measurement using the cross erosion and dilation element.

3.1.4.3 Repeat readings on the same kernels

As the video image was a live image, it could have been influenced by minute fluctuations in intensity caused by background noise such as electricity signals. Measurements were made in triplicate on the same kernels to determine the precision of the instrument. The standard deviation obtained gave an indication of the precision of the results. Pictures were taken of each step during the detection and IA procedure. This was followed by analysis of variance on the triplicate measurements on three kernels in order to determine the precision of the measurements. Tukey's honest significant difference (HSD) test was done using SAS PROC GLM procedures.

3.1.4.4 The effect of circle size on kernel illumination

Triplicate readings on three white kernels measured with four different circle sizes were done. Kernels were analysed as shown in Figure 3.2. Analysis of variance was done followed by paired comparison tests (Tukey's HSD). Tests were also done for interactions. The objective was to determine if a change in circle size would result in comparative changes in translucent area sizes for different kernels. If the circle size was increased at a fixed ratio, it was

expected that the detected area size would also increase at a similar ratio. It was expected that this ratio increase would be the same for all kernels. A significant interaction between individual measured kernels and circle size would have meant that additional variables such as the kernel size and thickness had a significant effect on the accurate detection of translucency in maize.

3.1.5 Optimisation of measurements

Measurements were optimised to allow for a fixed ratio between circle area and maize kernel area. Before a formula could be developed for calculating all maize kernel areas and adjusting detected translucent areas to produce values for fixed circle area/kernel area ratios, a series of calibration curves had to be developed. All maize kernels differ in size and shape and the size of a kernel would have an additional effect on the detected intensity of the translucency. In practise, it is not possible to adjust gray levels to allow for individual kernel size and shape and therefore, a fixed circle size was selected followed by the development of a calibration formula to adjust readings obtained for each kernel to a fixed circle area/kernel area ratio. The actual detected translucent area for a maize kernel was expected to be influenced by the following variables:

- Proportion of vitreous endosperm
- Size of the maize kernel
- Ratio of the circle area to the projected kernel area (bigger kernels will absorb more light to give lower readings)
- Thickness of the maize kernel
- Colour of the maize kernel.

3.1.5.1 Calibration of the fixed circle method of light exposure with the modeling clay method

Using modelling clay to surround each maize kernel on a light box, effectively produces a "circle" area/kernel area ratio of one, as the "circle" area is the same as the kernel area. The "circle" in this case was not round, but the same shape as the kernel area. This is the most correct method, but it is impractical to implement on a large scale, as it would require each kernel to be embedded individually with clay. By using a fixed sized circle slightly smaller than the kernels, with kernels placed on the circle all light was shone through the kernels. However, as the area of the circle was fixed, only a fixed amount of light shone through and kernels with larger areas gave lower readings than they should. Kernels with smaller areas gave higher readings than they should have. A calibration curve was therefore developed in order to allow all readings on all kernels to be adjusted to a fixed circle area/kernel area ratio to produce comparable results to those possible from the modeling clay method.

Three white kernels of one F2 hybrid of a South African cultivar were used for this experiment. The areas of translucent endosperm in the three maize kernels were measured using modeling clay on the IA. Background light was excluded using the modeling clay. Each kernel was then measured again in triplicate using a range of 7 different sized light circles, as described in Table 3.2. Gray value ranges for image segmentation for the kernels illuminated through the circles in the paper mask were adjusted until the same sized (area) binary image masks were created as those produced by using modelling clay. These data were analysed statistically and used to develop a relationship between the intensity of the gray pixel levels and the ratio of the light circle area to the area of the kernel. The relationship was then used later to correct for the readings on each individual kernel based on the kernel size and area.

Exposure percentage (EX) was calculated as follows:

$$EX = \frac{\text{Circle area } (mm^2)}{\text{Projected maize kernel area } (mm^2)} \times \frac{100}{1}$$

Analysis of variance was performed (LSD paired comparison test) to determine if the measured translucent areas were the same at every exposure percentage (EX). Eight exposure percentages (seven using fixed circles of different sizes and one using the modeling clay or kernel area) were tested and the hypotheses tested were:

Ho: T1=T2=T3=T4=T5=T6=T7=T8
Ha: T1≠T2≠T3≠T4≠T5≠T6≠T7≠T8.

T = Mean area of translucent endosperm at eight levels of EX.

The correlation coefficient (Pearson) between the EX and the determined minimum gray threshold detection level was determined following fitting a linear regression line to the data.

3.1.6 Translucency correction factors

3.1.6.1 Corrections for exposure

Three intact kernels were taken from the middle section of one cob from each of the cultivars for light intensity tests. In order to have the same number of white and yellow kernels for the analysis, three additional yellow kernels were taken from cultivars 7 and 8 (Table 3.1). The maize kernels were analysed in groups of three per cultivar. Each set of three kernels were subjected to light exposure using four different sets of circles, according to the method described in section 3.1.2.

The areas of the detected translucent parts were used for the calculation of the increase in translucency at a constant gray threshold level, but with increased exposure. A constant gray threshold level was used for all measurements and the level selected was all gray pixels on levels 54–255 for white maize and a level of 44–255 for yellow maize.

Projected maize kernel area was measured after measuring the translucency and an algorithm for the two detections was programmed into the software (Leica QWin User Guide 1996). The increase in translucency as a function of increased circle size in relation to projected kernel area size was calculated as follows for each exposure area bigger than the reference (for the same maize kernel):

The translucent area increase (TI) was expressed as a percentage as follows:

$$TI = \frac{(T_l - T_r)}{T_r} \times \frac{100}{1}$$

T_l = Translucent area at larger circle area

T_r = Translucent area at reference circle area

With the above formula, the translucent area increase (TI) will have a value of 0 for the reference exposure area. Calibration curves were plotted for all five cultivars with TI (Dependent variable) and EX (Independent variable). Linear regression lines were fitted for each cultivar followed by an adjustment in order to have a fixed EX at a zero point TI of 0%. With surrounding the kernels with modeling clay, 100% of the projected kernel area was exposed to light and these measurements were used as reference points at 100% EX. After the adjustment, the data were combined to produce a calibration curve. The resulting regression lines were used for future correction of translucent area measurements on maize kernels placed on fixed-sized light circles in the paper mask. Corrections were achieved by measuring the projected areas of individual maize kernels produced from a reflected light image followed by adjusting the size (area) of the detected binary translucent area mask images accordingly using the regression line. These calculations therefore took into account the size effect of maize kernels on the intensity of the transmitted light

images. Based on the optimum circle size as shown in Fig 3.2, a fixed circle of size 29.55 mm^2 was selected for further calculations.

Corrections for exposure were made to measure all kernels at a fixed ratio of circle area vs. projected kernel area using the following formulas:

$$\text{True translucent area } (mm^2) = \frac{\text{Thickness adjusted translucent area } (mm^2)}{\text{Correction factor for exposure}}$$

Correction factor for exposure = 1 + (TI/100), where TI = (4.02 x EX) – 55 (white maize), and EX = (circle area (mm^2)/Total kernel area (mm^2)) X 100.

Thickness adjustment is discussed in section 3.1.6.2.

The relationship between TI and EX for white maize was determined by linear regression of the calibration curve where TI = 4.02 EX – 55.
($r = 0.91$, $R^2 = 0.83$, $n = 60$, $p < 0.001$).

The relationship for yellow maize was: TI = 3.58 EX – 47, $r = 0.90$, $R^2 = 0.81$, $n = 60$, $p < 0.001$.

The translucency percentages using the corrected translucency values were calculated as follows:

$$\text{Translucency 1} = \frac{\text{True translucent area } (mm^2)}{\text{Projected kernel area } (mm^2)} \times \frac{100}{1}$$

$$\text{Translucency 2} = \frac{\text{True translucent area } (mm^2)}{\text{Endosperm area } (mm^2)} \times \frac{100}{1}$$

Endosperm area (mm^2) = Projected kernel area (mm^2) – germ area (mm^2)

3.1.6.2 Corrections for kernel thickness

A thickness correction factor was calculated by selecting ten kernels from each cultivar, measuring the translucency and the corresponding kernel thickness. The same kernels were then sanded on the flat side opposite from the germ with fine sandpaper to reduce the thickness by 0.5 mm increments. Translucency was measured at each individual thickness. Sandpaper with a grit size of 1000 was used in order to produce a smooth polished kernel surface. The seed coat layers of the kernels were transparent as only maize cultivars with a clear pericarp were used. Kernels with a coloured pericarp due to fungal or other damage were not measured. Measurements on the sanded kernels were discontinued when the germ was exposed after removing successive layers of tissue. The percentage increase in translucency was calculated for each kernel using the non-sanded measurement as a basis. Data were combined and a linear regression line fitted to the data. The resulting thickness correction factor was then used to adjust translucency readings together with the Exposure percentage (EX). Separate values were determined for white and yellow maize. The thickness of the kernels was measured by standing kernels on their sides and the distance between the top and bottom edges measured using Image Analysis. The average of five thickness measurements spaced evenly along the longitudinal axis of each kernel was calculated. Forty nine kernels were measured per cultivar and the mean value of each cultivar used for the thickness correction calculations.

3.1.7 Vitreousness determinations on single kernels (mass fraction)

3.1.7.1 Hand dissection of maize kernels

As the maize kernels were too hard for any of the dissection methods described in the literature (Louis-Alexandré, Mestres and Faure 1991; Yuan and Flores 1996; Dombrink-Kurtzman and Knutson 1997), a modified method was developed. Intact maize kernels (49) from each of the eight cultivars

were used. Each individual maize kernel was weighed (four decimal places precision). After weighing, the kernels were subjected to IA to measure the translucency and thickness and the values were adjusted according to the calibration curves. Each kernel was numbered after IA using a water resistant black marker pen from no 1 – 49 with the number corresponding to the number allocated by the Image Analyser. The numbered kernels were placed in plastic containers and filled with distilled water. The containers were sealed and the kernels soaked at 4°C for five days. After soaking, each kernel was weighed after excess water had been removed from the kernels using soft tissue paper. After weighing, each kernel was sectioned open longitudinally using a scalpel to obtain two flat halves. The two halves were then brushed with a Jordan V toothbrush (Junior size) under running water to remove all visible opaque (mealy) endosperm. Any opaque endosperm that could not be brushed out, was carefully scraped out using a scalpel.

After removal of opaque endosperm, the kernels were again weighed (the two halves together) after excess water had been removed using soft tissue paper. After weighing, the vitreous endosperm, germ and bran were separated using a scalpel. The vitreous endosperm was weighed after the separation. Vitreous endosperm was determined by weighing the actual dissected vitreous portions of each kernel after cleaning, while opaque endosperm was determined as the mass difference after brushing. The moisture content of each cultivar was determined before soaking on maize kernels from the same cob of which the dissected kernels were obtained using AACC method 44-18 two-stage drying (American Association of Cereal Chemists 2000). Moisture contents varied from 10.1 to 12.9%.

3.1.7.2 Calculation of the yield of vitreous and opaque endosperm

The dry masses of all weighed fractions of each maize kernel were calculated using the moisture content data obtained from the determination on the cultivar before soaking. The moisture content after soaking was calculated for

each individual kernel using the weight increase of the kernel after soaking. It was assumed that the moisture contents of the kernels after brushing and the vitreous endosperm after dissecting was the same as that of the whole kernel after soaking. To reduce the possibility of moisture loss during dissection, each kernel was dissected and weighed before the next kernel was taken out of the soaking water. The brushing of the kernels under running water also helped to reduce moisture loss during dissection. The yield of vitreous and opaque endosperm for each individual kernel was calculated as:

$$\text{Vitreous endosperm (\%)} = \frac{\text{Dry mass of vitreous endosperm}}{\text{Dry mass of whole kernel}} \times \frac{100}{1}$$

$$\text{Opaque endosperm (\%)} = \frac{\text{Dry mass of whole kernel} - \text{dry mass after brushing}}{\text{Dry mass of whole kernel}} \times \frac{100}{1}$$

The above calculations were also done without corrections for moisture.
Significant differences between different cultivars were tested using the Kruskal-Wallis (Keller and Warrack 2000) test for non-parametric data where sample residuals did not show normality, otherwise analysis of variance was done.

3.1.8 Statistical calculations and the development of regression models between translucency and endosperm yields

Analysis of variance and Tukey's HSD were performed on the results where applicable. The Kruskal Wallis test was performed on data where residuals did not show normality. Pearson correlation coefficients were determined followed by linear regression where applicable. The analysis were done in SAS (SAS 1989) using PROC REG, PROC GLM, PROC UNIVARIATE ((test for normality) and PROC CORR. All data were considered significant at $p < 0.05$. Data with smaller p-values (higher significance) were labeled accordingly. Z-transformations were done for the Pearson correlation

coefficients in order to test whether changes in the correlation coefficients were significant. Data were interpreted according to the tables provided by Diem and Seldrup (1982).

Correlations and regression calculations were done and levels of significance were calculated. Slopes for the regression models fitted were also tested for significance. Residuals of IA data were normally distributed and therefore no significant differences existed between mean and median values of the data hence only the mean and standard deviation values were calculated for all data. The correlation coefficient r was determined as a measure of the stochastic dependence of the dependent and independent variables used for determining relationships between IA measurements and milling yield data. A standard error of the regression slope was calculated along with a t-test to determine if the slope differed significantly from the horizontal or not. With this specific test, conclusions can be made on the data independent of the units of the graphs on the axes. A relationship may have a significant slope, but may seem flat on a line diagram because of the units of measurements or *vice versa*. Single tailed correlations and t-tests were used throughout the experimental work as the relationships tested were predicted for example higher translucency values were expected to predict a higher yield of vitreous endosperm. The exposure only corrections were not shown in the results, only the thickness corrections and the combined effects of thickness and exposure.

3.2 RESULTS

3.2.1 Image set-up

Pictures of the various stages in the procedure used for capturing images of vitreous endosperm in maize are shown in Figure 3.3.

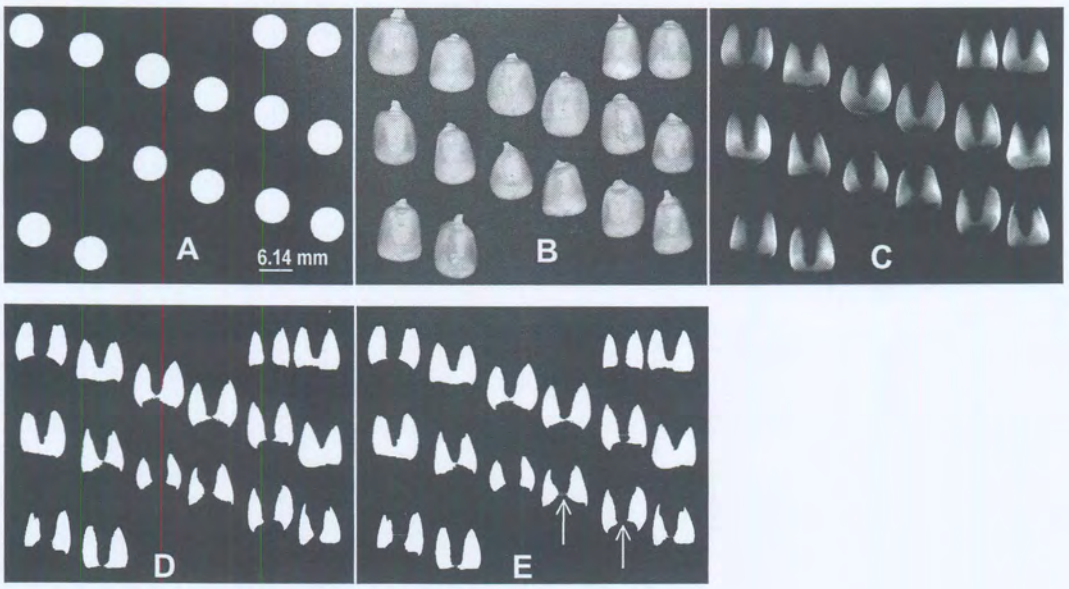

Figure 3.3 The appearance and detection of maize kernels on the light box. A, appearance of circles with light source below before maize kernels were placed on top; B, intact kernels with illumination from underneath and above; C, translucent endosperm visible with illumination from underneath through the paper mask; D, computer generated detected areas covering the translucent parts generated in C; E, paired sections of the areas connected using the vector element (thin connecting lines – see arrows) in order for the computer to calculate the areas as a combined single area for a kernel. All pictures are at the same magnification

3.2.2 Effect of humidity exposure on the detected translucent area of intact maize kernels

The results of the translucency measurements done on three kernels of three white maize cultivars before and after exposure to a relative humidity of 98% are shown in Table 3.3.

Table 3.3 The effect of air relative humidity on the detected translucent area (mm^2) measured in three white maize cutivars

Treatment	Cultivar 1	Cultivar 2	Cultivar 3
Before humidity exposure	79.22 (4.76)[a]**	81.34 (6.43)[b]*	49.82 (6.96)[c]
After humidity exposure	75.62 (3.17)[a]	82.62 (2.71)[b]	48.46 (8.89)[c]

* Mean and standard deviation

** Different superscripts in columns indicate statistically significant differences ($P<0.05$)

Results show that no significant differences existed between detected translucent areas of kernels before and after humidity exposure (Table 3.3).

3.2.3 Effects of translucency measurement methodology

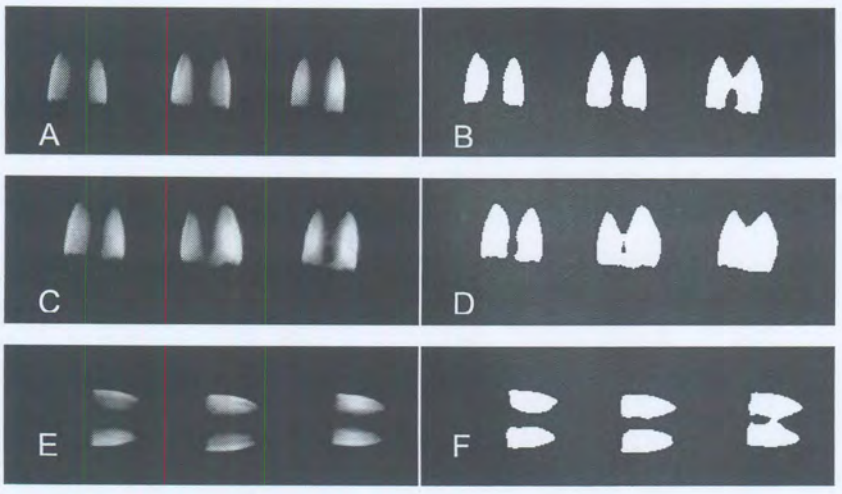

Figure 3.4 The effect of changing circle size on the appearance and size of the translucent area of the same maize kernels. A, captured image for three maize kernels, circle size at 29.5 mm^2; B, detected pixel area at circle size of 29.5 mm^2 (gray threshold between 54 and 255); C, captured image for the same three maize kernels, circle size at 43.77 mm^2; D, detected pixel area at circle size of 43.77 mm^2 (gray threshold between 54 and 255); E, similar to A, but at a different orientation; F, similar to B, but at a different orientation

The effects of changing circle size on the translucent area detected and the effect of kernel orientation are illustrated in Figure 3.4. The larger circle size (C and D) caused larger binary images if pixels were detected in the same intensity range as compared to smaller circle size (A and B). Kernel orientation had no effect on the shape and size of the binary images.

Measurements of the detected translucent areas at constant gray levels at four different circles are given in Table 3.4. The effect of binary amendment is shown graphically in Figure 3.5.

Table 3.4 The effect of circle size on the detected translucent area (mm^2) at constant gray level and constant amendment for three white maize kernels measured at each of four circle sizes

Circle size (mm^2)	**Kernel 1**	**Kernel 2**	**Kernel 3**
17.2	34.72 (1.49) [a*]	33.75 (1.15) [a**]	38.52 (0.19) [e]
29.6	57.04 (0.36) [b]	52.46 (0.37) [f]	62.11 (0.27) [g]
43.8	78.12 (1.25) [c]	71.22 (1.02) [h]	78.79 (0.93) [c]
48.3	88.86 (0.92) [d]	83.33 (0.37) [j]	84.05 (0.85) [i]

* Mean and standard deviation of triplicate measurement on each kernel

** Different superscripts in rows as well as columns refer to statistically significant differences (Tukey HSD test, paired comparisons, $p < 0.001$).

The results in Table 3.4 show that with increased circle size, the detected translucent area also increased for each kernel due to the increased amount of light entering the kernels. There was also small variation in the detected size of the same kernel with the same circle size.

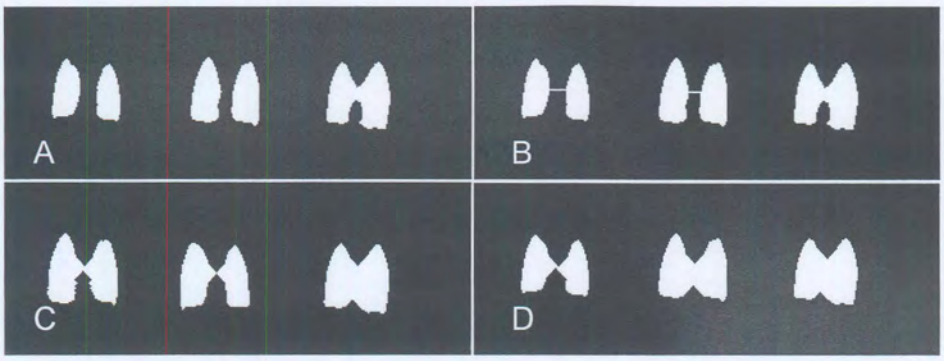

Figure 3.5 Comparisons between different techniques of combining detected surface areas earmarked for measurement. A, detected binary; B, correcting areas using the vector function; C, connecting areas using the amend (dilation and erosion function – cross element); D, connecting areas using the amend function (dilation and erosion function – octagon element)

The effect of kernel orientation and amendment method as illustrated in Figures 3.4 and 3.5 on the measured detected translucent area is shown in Table 3.5. Figure 3.5 clearly shows an effect of amendment method on the distortion of the binary image covering the detected translucent areas of the kernels. Amendment B (linking areas with a thin line) had the least amount of visible distortion to the areas.

Table 3.5 The effect of kernel orientation, binary amendment method and repeat analysis on the detected translucent area (mm^2) of maize kernels

Description	Orientation (detected area, mm^2)	Description	Amendment (detected area, mm^2)	Description	Repeat Analysis (detected area, mm^2)
Vertical (90°)	58.9 (4.9) [a]*	No amendment***	58.6 (4.0) [a]	First analysis****	58.3 (4.8) [a]
Horizontal (0°)	58.1 (4.9) [a]***	Vector amendment	58.6 (3.9) [a]	Second analysis	58.9 (5.2) [a]
		Dilation and erosion amendment	62.5 (4.3) [b]	Third analysis	58.3 (4.9) [a]

* Mean and standard deviation

** Different superscripts in columns refer to statistically significant differences ($P< 0.01$)

*** No amendment (manual adding of detected areas)

**** Repeat analysis was done on the same set of kernels

Kernel orientation had no effect on the detected translucent areas (Table 3.5). Amendment method did have a significant effect on the detected translucent areas. The vector amendment did not increase the measurements significantly, but the dilation and erosion amendment did increase the measurements significantly (as was also visible in Figure 3.5). Repeat analysis produced results which were not significantly different from each other, indicating that the set-up of the measurement system as described was stable and precise after the initial settings were completed.

3.2.4 Optimisation of measurements

Table 3.6 Comparison between the mean detected translucent areas (mm^2) of maize kernels of different sizes (three kernels per size) measured using a constant circle (29.6 mm^2) and modeling clay for light exposure

Size of kernels**	**Size 1 (mm^2) 96.9 (3.1) ***	**Size 2 (mm^2) 154.7 (9.9)**	**Size 3 (mm^2) 104.7 (2.6)**	**Size 4 (mm^2) 77.9 (2.3)**
Circle (29.6 mm^2)	29.8 (1.7) [a**]	72.9 (6.4) [b]	85.2 (0.1) [c]	0.32 (0.17) [d***]
Modeling clay	29.7 (3.0) [a]	86.7 (5.5) [c]	85.4 (2.7) [c]	0.16 (0.10) [d***]

* Mean and standard deviations

** Different superscripts in rows as well as columns indicate statistically significant differences (LSD analysis of variance, $p<0.05$).

*** These kernels had very low levels of translucency (they were almost completely opaque).

**** The size of the kernels was the total projected area of a kernel laying flat as detected by the camera, three kernels of similar size were selected for each size group and standard deviations show size variation between three kernels in the group

The results in Table 3.6 show that different area sizes were detected for size 2, but the same areas were detected for the other sizes when comparing the two methods (modeling clay vs. fixed circle). This indicated that the areas detected using modeling clay cannot be repeated using a fixed circle for illumination and other unidentified factors were present that influenced the results.

The effect of the exposure percentage (EX) on the gray threshold detection level to produce the same translucency area on the same maize kernels at eight different exposure ratios is shown in Table 3.7. A plot of the measurements is shown in Figure 3.6 with a linear regression line fitted. The Pearson correlation coefficient was determined for the data followed by a fitted linear regression line.

Table 3.7 The effect of exposure percentage (EX) on the gray threshold detection level necessary to produce the same translucent area (mm^2) on the same maize kernels

Description	Setting 1*	Setting 2	Setting 3	Setting 4	Setting 5	Setting 6	Setting 7	Setting 8***
Circle area (mm^2)	17.2 (0.2) [a]**	29.6 (0.6) [b]	43.8 (0.7) [c]	48.3 (0.6) [c]	66.2 (0.9) [d]	88.4 (0.9) [e]	113.4 (0.9) [f]	154.6 (1.0) [g]
Exposure percent EX (%)***	11.3 (0.9) [a] ******	19.2 (1.5) [b]	28.4 (2.2) [c]	31.3 (2.1) [c]	42.9 (3.2) [d]	57.3 (3.5) [e]	73.5 (4.3) [f]	100 (0.0) [g]
Gray threshold for detecting the same translucent area at each circle size	9****	32	60	69	97	144	168	244
Actual detected translucent area (mm^2) at each circle	86.5 (2.1) [a]	86.6 (5.7) [a]	86.7 (2.5) [a]	86.7 (2.7) [a]	86.5 (6.0) [a]	86.4 (5.1) [a]	86.6 (6.5) [a]	86.7 (5.5) [a]

* Setting number using the same three maize kernels (projected kernel area was 154.6 (1.0) mm^2 in all instances as the same kernels were used)

** Different superscripts within rows indicate statistically significant differences (LSD Analysis of variance, $p < 0.05$)

*** Setting 8 = modeling clay with an exposure percentage of 100 (kernel area/kernel area x 100)

**** The same threshold level was used for the three kernels within each group (threshold levels were not adjusted for individual kernels as well)

***** EX = (Circle area/Total kernel area) x 100

****** Mean and standard deviation

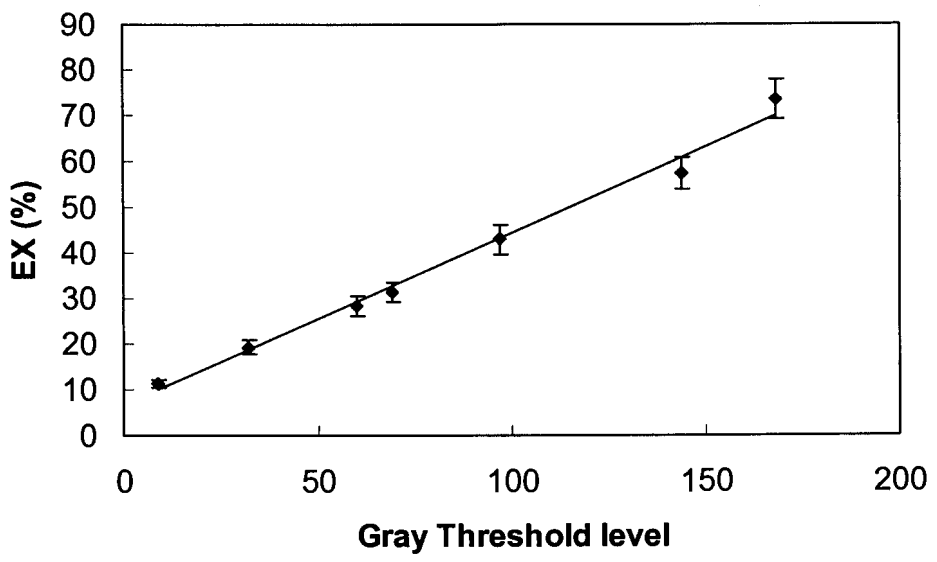

Figure 3.6 The relationship between gray threshold detection level and exposure percentage (EX) when three intact white maize kernels were measured at seven different circle sizes. The gray threshold levels were set for each EX at such a level that the same translucent area on the kernels was detected (see Table 3.7). $r = 0.99$, $y = 0.37x + 6.53$, $R^2 = 0.98$

3.2.5 Optimisation of translucency correction factors

3.2.5.1 Corrections for exposure

The effect of EX on TI using three maize kernels of one cultivar is shown in Figure 3.7. After adjusting the data to refit the regression line at a TI of 0% and an EX of 15%, the new regression line is shown in Figure 3.8. The EX of 15% was chosen as the smallest circle size relative to the kernel size. Below this value, kernels were unevenly illuminated.

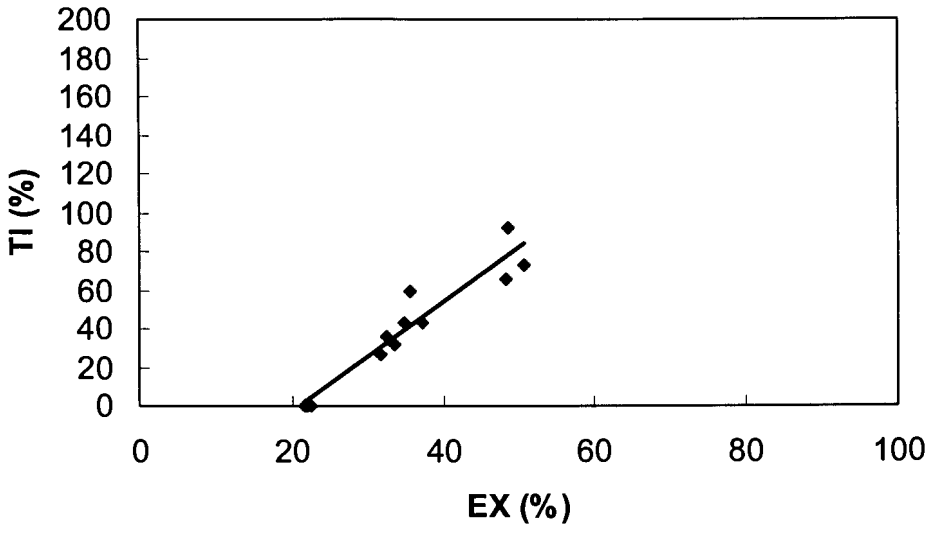

Figure 3.7 The effect of exposure percentage (EX) on the translucency increase percentage (TI) of maize kernels of cultivar 1, without a fixed zero TI correction, $y = 2.8x - 59.0$, $R^2 = 0.92$

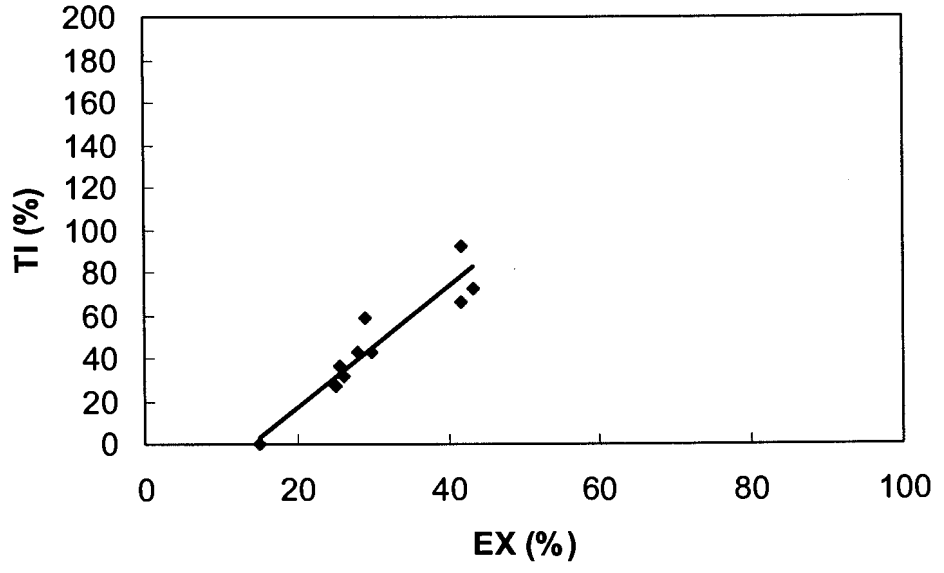

Figure 3.8 The effect of exposure percentage (EX) on the translucency increase percentage (TI) of maize kernels of cultivar 1, with a fixed zero TI correction at an EX of 15%, $y = 2.8x - 39.8$, $R^2 = 0.92$

The slope and R^2 of each fitted line for each cultivar at an EX of 15% and TI of 0% is summarized in Table 3.8.

Table 3.8 Regression data for fitted linear regression lines for EX and TI of eight maize cultivars after adjustment to an EX of 15%

Cultivar number	Slope	Pearson r	R^2
1 (SR 52)	2.83	0.96*	0.92
2 (L290)	4.19	0.98*	0.97
3 (CRN 429)	4.95	0.97*	0.91
4 (R 827)	3.36	0.96*	0.94
5 (CRN 439)	4.63	0.95*	0.92
6 (N282)	2.56	0.99*	0.99
7 (N290)	4.41	0.94*	0.92
8 (N258)	3.82	0.88*	0.85

* $p<0.0001$ for all relationships

The correlation between EX and TI was highly significant for each individual cultivar (Table 3.8). Small differences existed in the slope of the lines of the cultivars (Table 3.8), but after combining all the results, the relationships were still highly significant with high correlation coefficients (Figures 3.9 and 3.10). The values for white and yellow maize kernels were not combined because white and yellow maize translucent areas were detected at different gray thresholds. The reason for the difference was the slightly darker image obtained when the yellow kernels were converted to a monochrome image than the white kernels. Similar correlation and regression analyses were done for the remaining 7 cultivars and in all cases adjustments were made to allow for a fixed zero point EX of 15% at a TI of 0%. After adjusting the data to refit the regression line at a TI of 0% and an EX of 15%, the regression line is shown in Figure 3.8. The EX of 15% was chosen as the smallest circle size for a maize kernel relative to the kernel size. Below this value, maize kernels

tended to become unevenly illuminated which could influence the results of the translucency measurement.

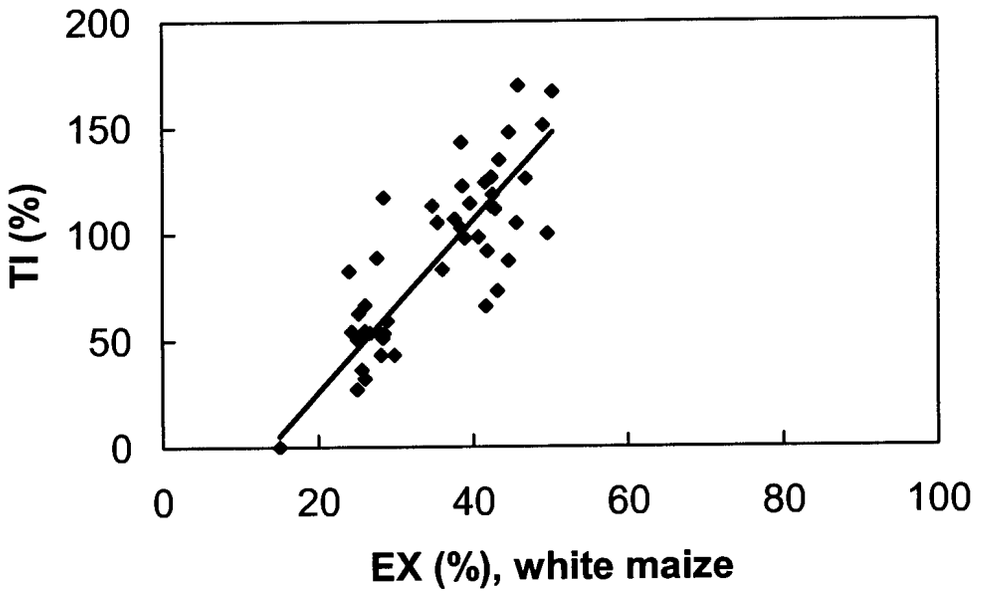

Figure 3.9 The effect of exposure percentage (EX) on the translucency increase percentage (TI) of five combined white maize cultivars. $y = 4.02x - 55$, $r = 0.91$, $R^2 = 0.83$

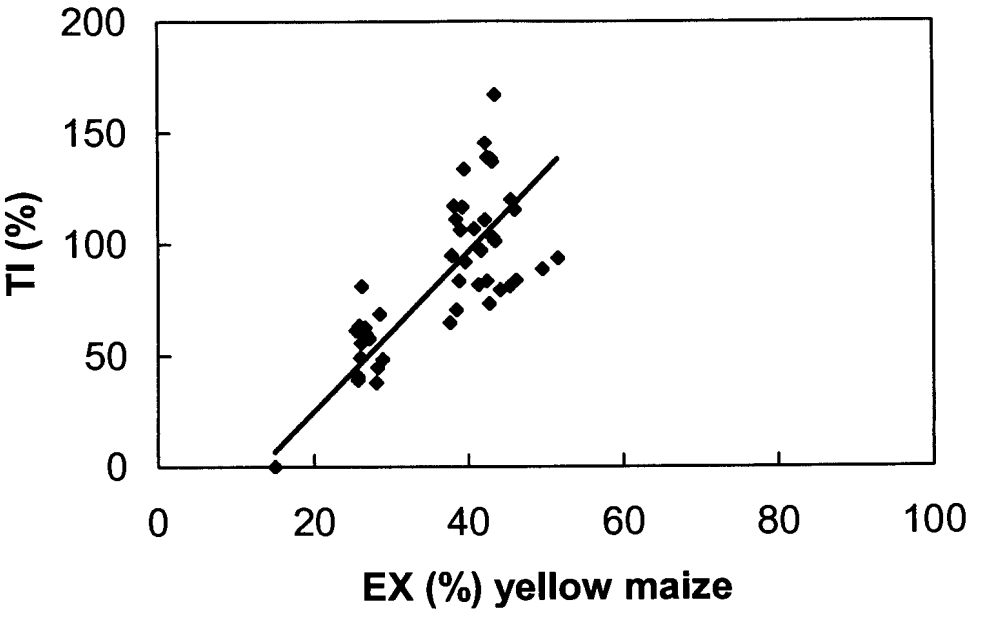

Figure 3.10 The effect of exposure percentage (EX) on the translucency increase percentage (TI) of three combined yellow maize cultivars. $y = 3.58x - 47$, $r = 0.90$, $R^2 = 0.81$

3.2.5.2 Corrections for kernel thickness

The relationships between the thickness of maize kernels and the change (%) in the detected translucent area (mm^2) at constant gray threshold and EX levels were linear (Figures 3.11 and 3.12). Both linear regressions were highly significant ($p < 0.001$). The thickness factors for white and yellow maize were the same.

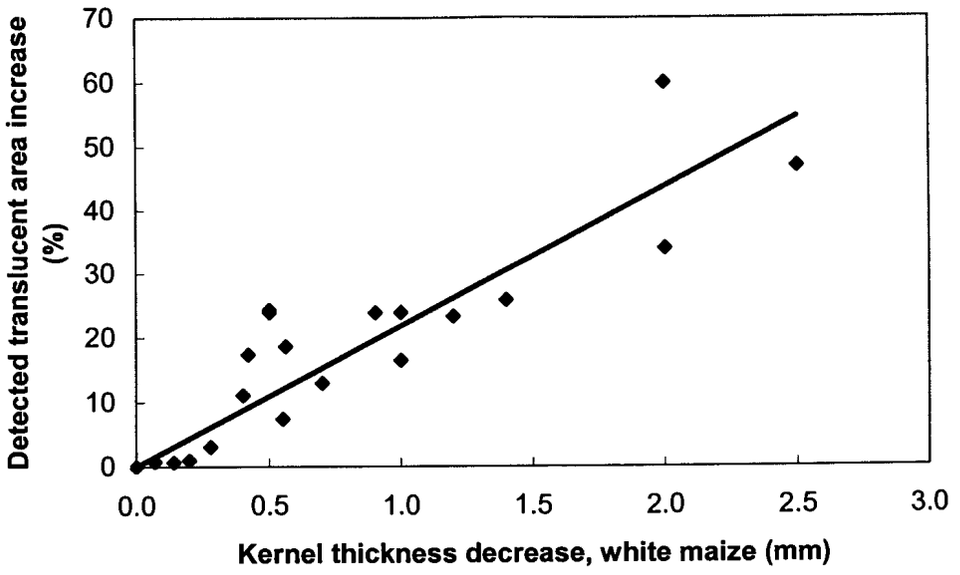

Figure 3.11 The effect of white maize kernel thickness decrease (mm) on the detected translucent area increase (%). y = 21.86x, r = 0.89, R^2 = 0.78

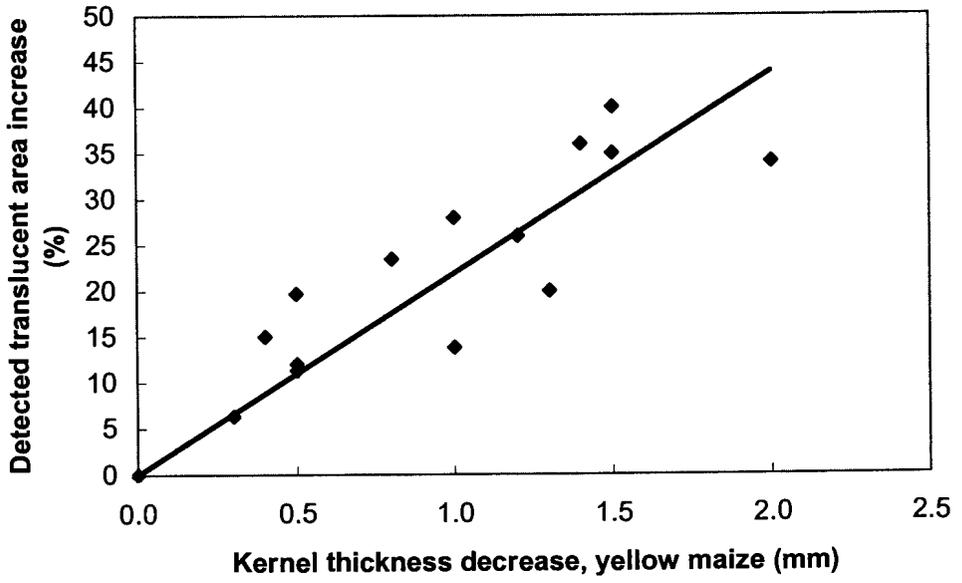

Figure 3.12 The effect of yellow maize kernel thickness decrease (mm) on the detected translucent area increase (mm). y = 21.94x, r = 0.88, R^2 = 0.74

3.2.6 Vitreousness measurements on single kernels (mass fraction)

3.2.6.1 Hand dissection of maize kernels

Images showing the dissection procedure are given in Figure 3.13.

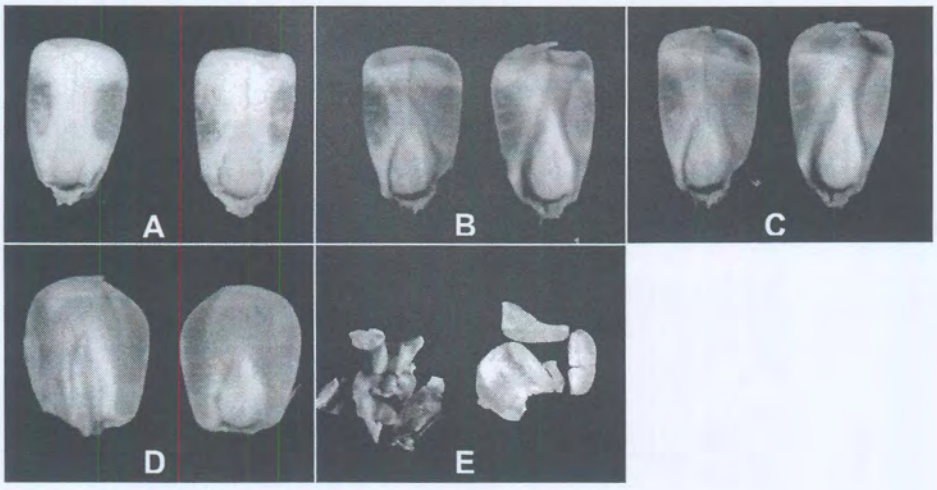

Figure 3.13 Longitudinal sections cut using a scalpel of yellow and white dent maize kernels after soaking in water for five days at 4°C. A, vitreous and opaque endosperm, yellow kernels; B, partial removal of the opaque endosperm with a toothbrush, yellow kernels; C, removal of all opaque endosperm after scraping with a scalpel followed by brushing, yellow kernels; D, similarly cleaned white kernel (all opaque endosperm removed by brushing and scraping); E, the dissected morphological parts of the white kernels

3.2.6.2 Calculating the yield of vitreous and opaque endosperm

Table 3.9 Hand dissection measurements on 49 kernels of five F2 white maize hybrids

Cultivar		Kernel mass (g)	Vitreous endosperm (g)	Opaque endosperm (g)	Vitreous (%)	Opaque (%)
1 (SR52)	Mean	0.46	0.21	0.14	46.3 [a*]	31.3 [a**]
	St. Dev	0.03	0.02	0.02	3.2	3.3
	Median	0.46	0.21	0.14	46.2	31.2
2 (L390)	Mean	0.39	0.25	0.06	63.1 [b*]	15.7 [b]
	St. Dev	0.04	0.02	0.02	3.0	3.4
	Median	0.39	0.25	0.06	63.7	15.2
3(CRN 429)	Mean	0.45	0.25	0.10	55.8 [c*]	22.0 [c]
	St. Dev	0.03	0.02	0.02	2.8	2.9
	Median	0.44	0.25	0.10	55.5	21.6
4 (R827)	Mean	0.34	0.18	0.09	53.2 [d*]	26.6 [d]
	St. Dev	0.05	0.03	0.02	3.2	2.8
	Median	0.32	0.17	0.09	52.9	26.2
5(CRN 439)	Mean	0.39	0.21	0.09	54.0 [d*]	23.8 [e]
	St. Dev	0.04	0.02	0.02	3.5	4.1
	Median	0.39	0.21	0.09	54.1	23.4

Vitreous (%) – vitreous endosperm % of total kernel mass (moisture free basis)
Opaque (%) – opaque endosperm % of total kernel mass (moisture free basis)

* Kruskal-Wallis test
** Different superscripts in columns refer to statistically significant differences ($P < 0.05$)

Table 3.10 Hand dissection measurements on 49 kernels of three F2 yellow maize hybrids

Cultivar		Kernel mass (g)	Vitreous endosperm (g)	Opaque endosperm (g)	Vitreous (%)	Opaque (%)
6 (N282)	Mean	0.32	0.16	0.08	49.9 [a*]	26.3 [a**]
	St. Dev	0.03	0.05	0.03	13.4	10.2
	Median	0.32	0.17	0.08	55.3	23.4
7 (N290)	Mean	0.42	0.22	0.10	52.7 [b*]	23.1 [b]
	St. Dev	0.05	0.03	0.02	3.5	3.1
	Median	0.42	0.22	0.09	52.6	23.1
8(N258)	Mean	0.42	0.24	0.09	56.4 [b*]	22.4 [b]
	St. Dev	0.03	0.02	0.01	3.1	2.4
	Median	0.42	0.23	0.09	56.0	22.1

Vitreous (%) – vitreous endosperm % of total kernel mass (Moisture free basis)
Opaque (%) – opaque endosperm % of total kernel mass (Moisture free basis)

* Kruskal-Wallis test
** Different superscripts in columns refer to statistically significant differences ($P < 0.05$)

3.2.7 Statistical calculations and the development of correlations

3.2.7.1 IA measurements

Table 3.11 Image Analysis measurements on 49 kernels of five white F2 maize hybrids

Cultivar		Tra (mm^2)	Trb (mm^2)	Trc (mm^2)	Tr1 (%)	Tr2 (%)	Length (mm)	Width (mm)	Thickness (mm)	Total kernel area (mm^2)	Germ area (mm^2)
1 (SR52)	Mean	45.3	45.3	32.0	26.5[a*]	40.7[a*]	13.7	11.7	4.7[a**]	120.0	41.9
	St. Dev	9.3	9.3	6.6	5.5	8.3	0.5	0.3	0.6	6.3	3.6
	Median	43.5	43.5	31.2	25.2	38.9	13.6	11.7	4.6	120.0	41.9
2 (L390)	Mean	82.6	91.7	58.2	56.0[b*]	87.2[b*]	12.8	10.5	5.2[b]	104.0	36.2
	St. Dev	6.9	7.6	5.4	4.9	8.9	0.5	0.5	0.4	6.8	3.8
	Median	83.5	92.7	58.9	56.3	87.0	12.8	10.6	5.3	105.0	36.1
3 (CRN 429)	Mean	92.9	84.5	61.8	48.8[c*]	61.5[c*]	13.9	11.6	3.9[c]	127.0	26.0
	St. Dev	9.0	8.2	7.6	4.4	5.4	0.5	0.5	0.3	7.7	4.4
	Median	94.5	86.2	61.5	49.4	62.2	14.1	11.7	3.9	127.0	35.4
4 (R827)	Mean	83.5	76.2	45.5	47.6[d*]	59.4[d*]	12.6	9.9	3.9[c]	96.0	18.9
	St. Dev	6.4	5.8	2.3	4.5	6.7	0.5	0.6	0.5	8.8	3.8
	Median	82.8	75.5	45.3	47.0	58.3	12.8	9.9	3.7	95.0	18.2
5 (CRN 439)	Mean	84.6	77.2	50.4	46.7[d*]	57.4[d*]	12.6	11.2	3.9[c]	108.0	20.2
	St. Dev	8.3	7.6	6.6	4.1	4.7	0.4	0.3	0.4	6.0	2.9
	Median	84.1	76.7	50.0	46.4	57.7	12.5	11.2	3.9	108.0	20.6

Tra – translucent area without corrections
Trb – translucent area with thickness corrections
Trc – translucent area with thickness and exposure ratio corrections
Tr1% - Translucent area % formula 1 (section 3.4.2)
Tr2% - Translucent area % formula 2 (section 3.4.2)
* Kruskal-Wallis test
** Different superscripts in columns refer to statistically significant differences ($p<0.05$)

Table 3.12 Image Analysis measurements on 49 kernels of three yellow F2 maize hybrids

Cultivar		Tra (mm^2)	Trb (mm^2)	Trc (mm^2)	Tr1 (%)	Tr2 (%)	Length (mm)	Width (mm)	Thickness (mm)	Total kernel area (mm^2)	Germ area (mm^2)
6 (N282)	Mean	53.5	51.5	28.3	34.5[a*]	56.5[a*]	12.3	8.6	4.4[a**]	82.0	31.8
	St. Dev	18.7	18.0	10.1	12.0	19.8	0.5	0.35	0.5	4.1	3.1
	Median	60.0	57.7	31.7	38.4	63.2	12.4	8.5	4.4	82.3	32.1
7 (N290)	Mean	69.5	69.5	42.4	44.5[b*]	74.6[b*]	13.4	10.1	4.6[a]	95.4	37.9
	St. Dev	10.0	10.0	6.4	6.3	11.4	0.4	0.5	0.6	4.9	4.7
	Median	69.8	69.8	42.5	44.6	74.7	13.4	10.1	4.6	96.6	37.1
8 (N258)	Mean	80.4	80.4	49.3	51.4[b*]	79.4[b*]	12.6	10.8	4.6[a]	95.9	33.7
	St. Dev	7.2	7.2	5.0	4.4	7.6	0.3	0.3	0.4	4.1	3.4
	Median	80.6	80.6	48.8	51.4	79.2	12.5	10.8	4.5	95.2	33.6

Tra – translucent area without corrections
Trb – translucent area with thickness corrections
Trc – translucent area with thickness and exposure ratio corrections
Tr1% - Translucent area % formula 1 (section 3.4.2)
Tr2% - Translucent area % formula 2 (section 3.4.2)

* Kruskal-Wallis test

** Different superscripts in columns refer to statistically significant differences ($p<0.05$)

3.2.7.2 Correlations and optimisation of relationships

Table 3.13 Product moment correlation coefficient (r) and coefficient of determination (R^2) matrixes for white maize. N = 245 for each data set

	Transl.1[a] (no corrections)	Transl. 2[b] (no corrections)	Transl. 1 (thickness correction)	Transl. 2 (thickness correction)	Transl.1 (thickness and exposure corrections)	Transl. 2 (thickness and exposure corrections)
Vitreous[c] (mass %) r	0.60**	0.74***	0.75***	0.79***	0.77***	0.81***
Vitreous (mass %) R^2	0.36	0.55	0.55	0.63	0.59	0.65
Opaque[d] (mass %) r	-0.53*	-0.67**	-0.67**	-0.74***	-0.72***	-0.77***
Opaque (mass %) R^2	0.28	0.45	0.45	0.54	0.51	0.60

a Translucent area (% whole kernel)
b Translucent area (% of endosperm)
c vitreous endosperm mass % of whole kernel
d opaque endosperm mass % of whole kernel
* $p < 0.001$; ** $p < 0.0001$;*** $p < 0.00001$ for level of significance of the correlation coefficient (significantly different from 0).

Table 3.14 Product moment correlation coefficient (r) and coefficient of determination (R^2) matrixes for yellow maize. N = 146 for each data set

	Transl.1[a] (no corrections)	**Transl. 2[b] (no corrections)**	**Transl. 1 (thickness correction)**	**Transl. 2 (thickness correction)**	**Transl.1 (thickness and exposure corrections)**	**Transl. 2 (thickness and exposure corrections)**
Vitreous[c] (mass %) r	0.84***	0.80***	0.83***	0.79***	0.78***	0.76***
Vitreous (mass %) R^2	0.71	0.64	0.69	0.62	0.62	0.59
Opaque[d] (mass %) r	-0.76***	-0.76***	-0.75***	-0.75***	-0.71***	-0.72***
Opaque (mass %) R^2	0.58	0.58	0.57	0.57	0.51	0.52

a Translucent area (% whole kernel)
b Translucent area (% of endosperm)
c vitreous endosperm mass % of whole kernel
d opaque endosperm mass % of whole kernel
* $p < 0.001$; ** $p < 0.0001$; *** $p < 0.00001$ for level of significance of the correlation coefficient (significantly different from 0).

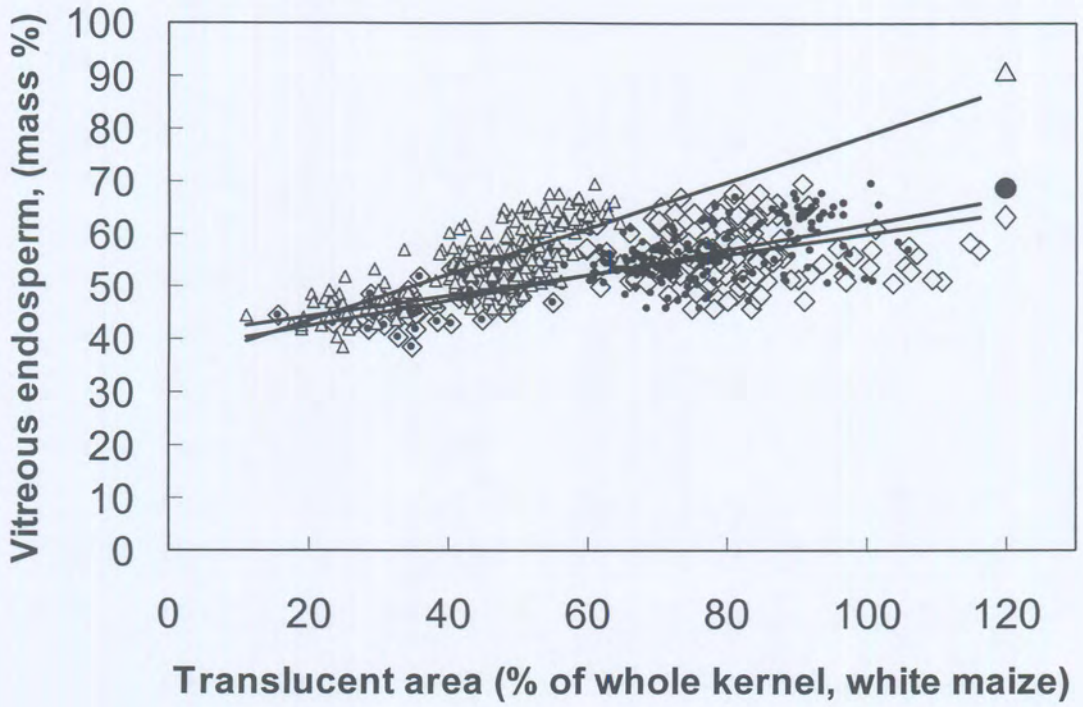

Figure 3.14 The effect of applying translucency correction factors (CFs) on the relationship between vitreous endosperm (mass %) as determined by hand dissection and the translucent area (% of whole kernel) of white maize as determined using IA. "◊", before any CFs (y = 0.19x + 41, r = 0.60); "●", after thickness CF (y = 0.24x + 38, r = 0.75) and "△", after thickness and exposure CFs (y = 0.44x + 35, r = 0.77), n = 245. r = 0.60 differed significantly (Fisher test) from r = 0.75 and r = 0.77 ($P<0.01$). r = 0.75 and r = 0.77 did not differ significantly from each other (Fisher test). Slopes were significantly different from the horizontal

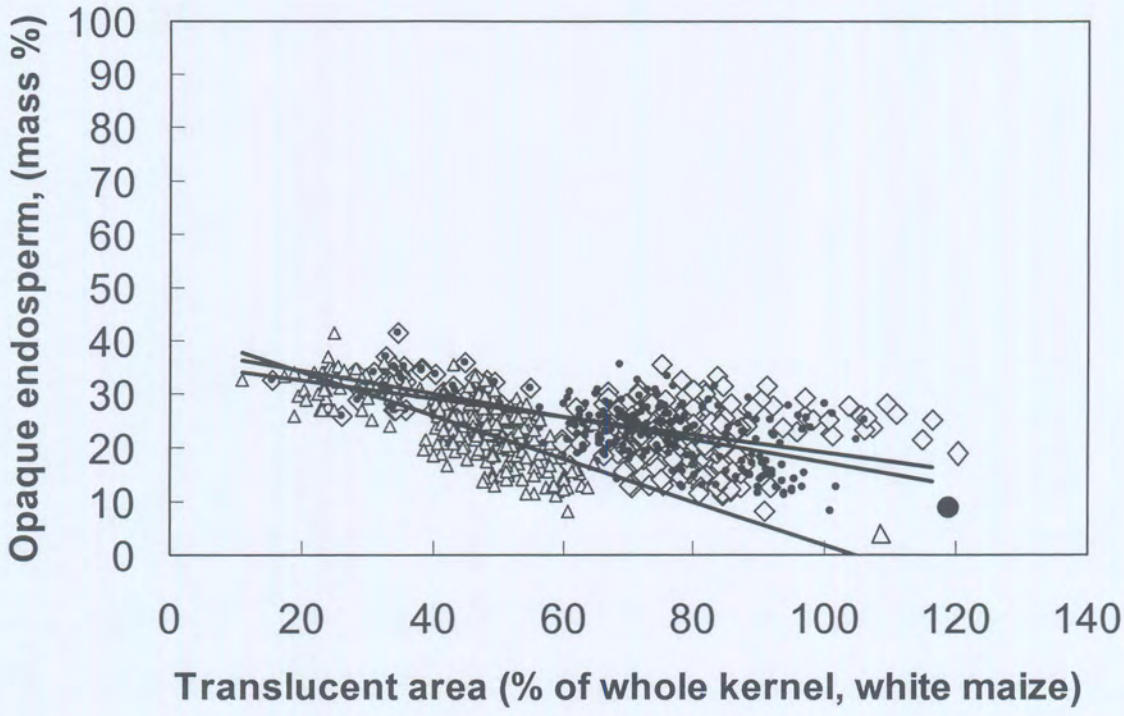

Figure 3.15 The effect of applying translucency correction factors (CFs) on the relationship between opaque endosperm (mass %) as determined by hand dissection and the translucent area (% of whole kernel) of white maize as determined using IA. "◊", before any CFs ($y = -0.17x + 36$, $r = -0.53$); "●" after thickness CF ($y = -0.22x + 39$, $r = -0.67$) and "△", after thickness and exposure CFs ($y = -0.40x + 42$, $r = -0.72$), $n = 245$. $r = -0.53$ differed significantly from $r = -0.67$ ($P<0.05$); $r = -0.53$ differed significantly from $r = -0.72$ ($p < 0.001$); $R = -0.67$ and $r = -0.72$ did not differ significantly from each other (Fisher test). Slopes differed significantly from the horizontal

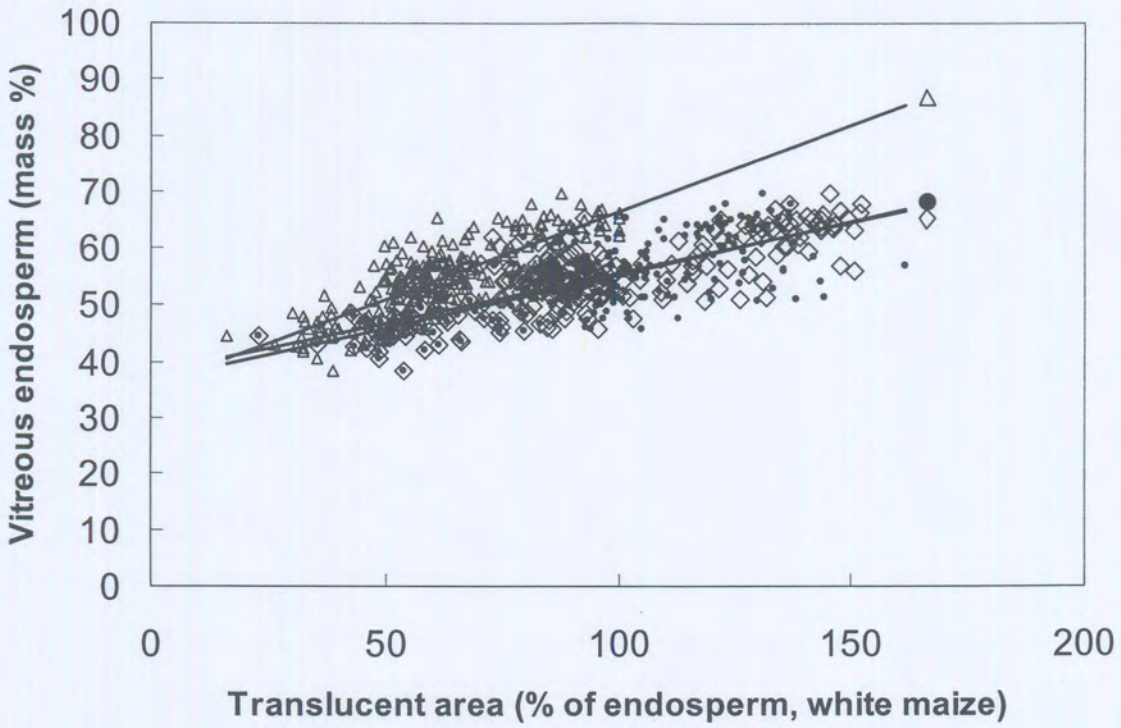

Figure 3.16 The effect of applying translucency correction factors (CFs) on the relationship between vitreous endosperm (mass %) as determined by hand dissection and the translucent area (% of endosperm) of white maize as determined using IA. "◊", before any CFs (y = 0.19x + 37, r = 0.74); "●", after thickness CF (y = 0.18x + 38, r = 0.79) and "△", after thickness and exposure CFs (y = 0.31x + 35, r = 0.81), n = 245. r-values did not differ significantly from each other (Fisher test). Slopes differed significantly from the horizontal

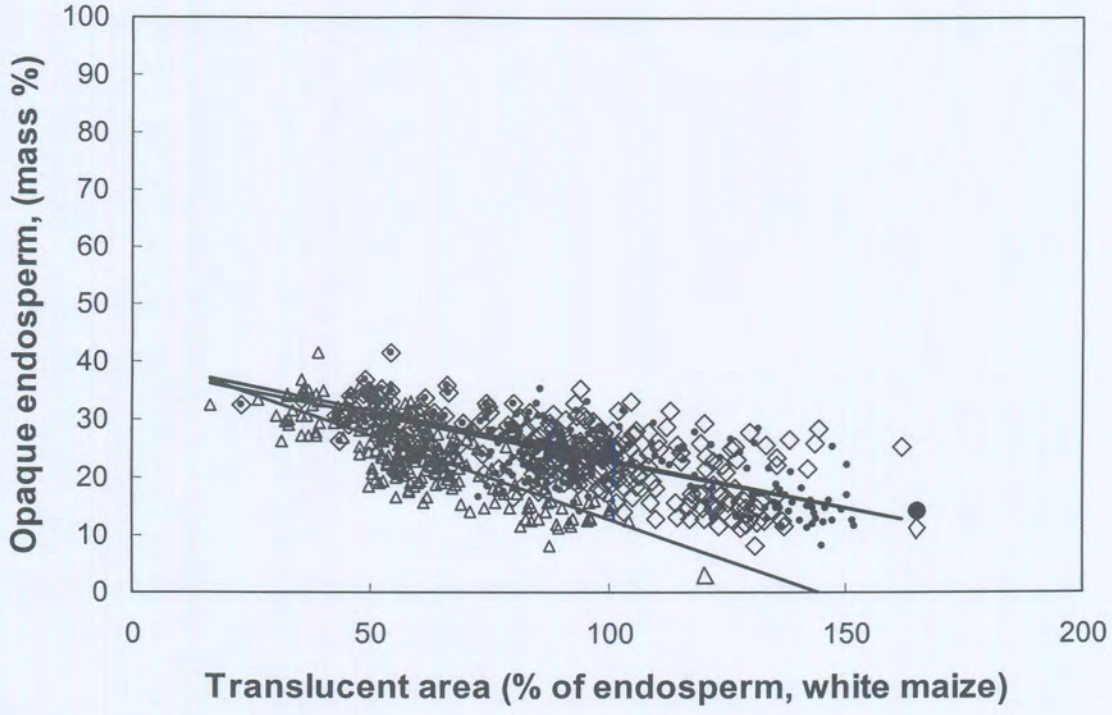

Figure 3.17 The effect of applying translucency correction factors (CFs) on the relationship between opaque endosperm (mass %) as determined by hand dissection and the translucent area (% of endosperm) of white maize as determined using IA. "◊", before CFs (y = -0.17x + 41, r = -0.67); "●", after thickness CF (y = -0.16x + 39, r = -0.74) and "△", after thickness and exposure CFs, (y = -0.29x + 42, r = -0.77), n = 245. r = -0.67 differed significantly from r = -0.77, but other r-values did not differ significantly from each other (Fisher test). Slopes differed significantly from the horizontal

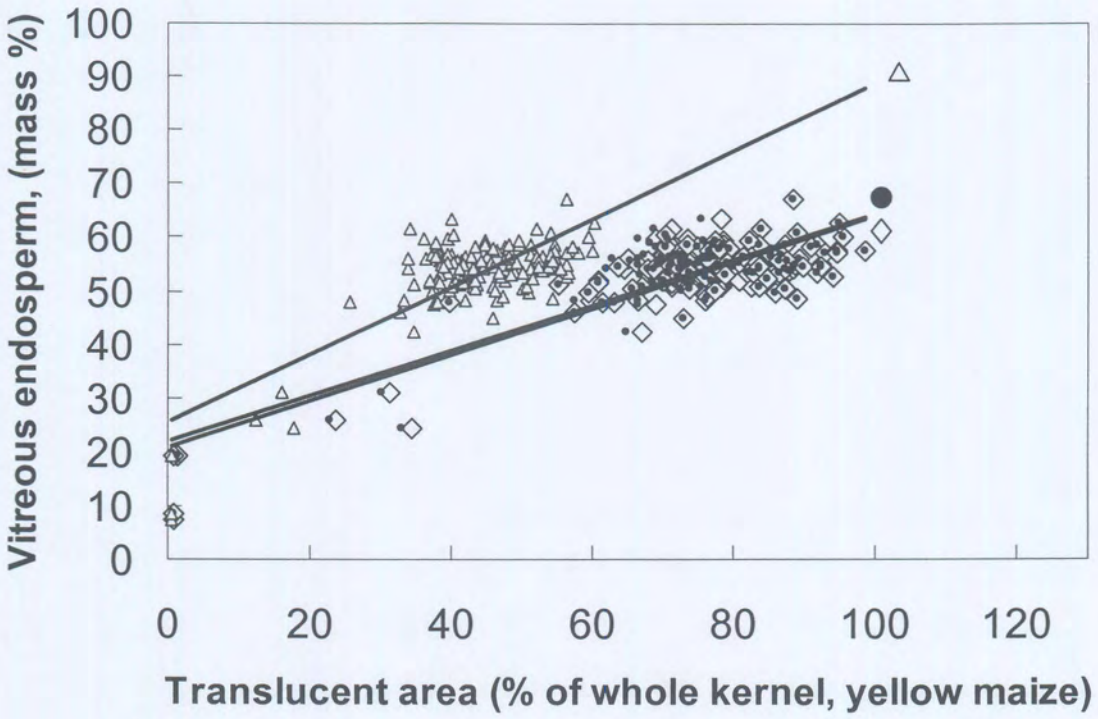

Figure 3.18 The effect of applying translucency correction factors (CFs) on the relationship between vitreous endosperm (mass %) as determined by hand dissection and the translucent area (% of whole kernel) of yellow maize as determined using IA. "◊", before CFs (y = 0.43x + 21, r = 0.84); "●", after thickness CF (y = 0.42x + 22, r = 0.83) and "△", after thickness and exposure CFs (y = 0.63x + 26, r = 0.79), n = 146. r-values did not differ significantly from each other (Fisher test). Slopes differed significantly from the horizontal

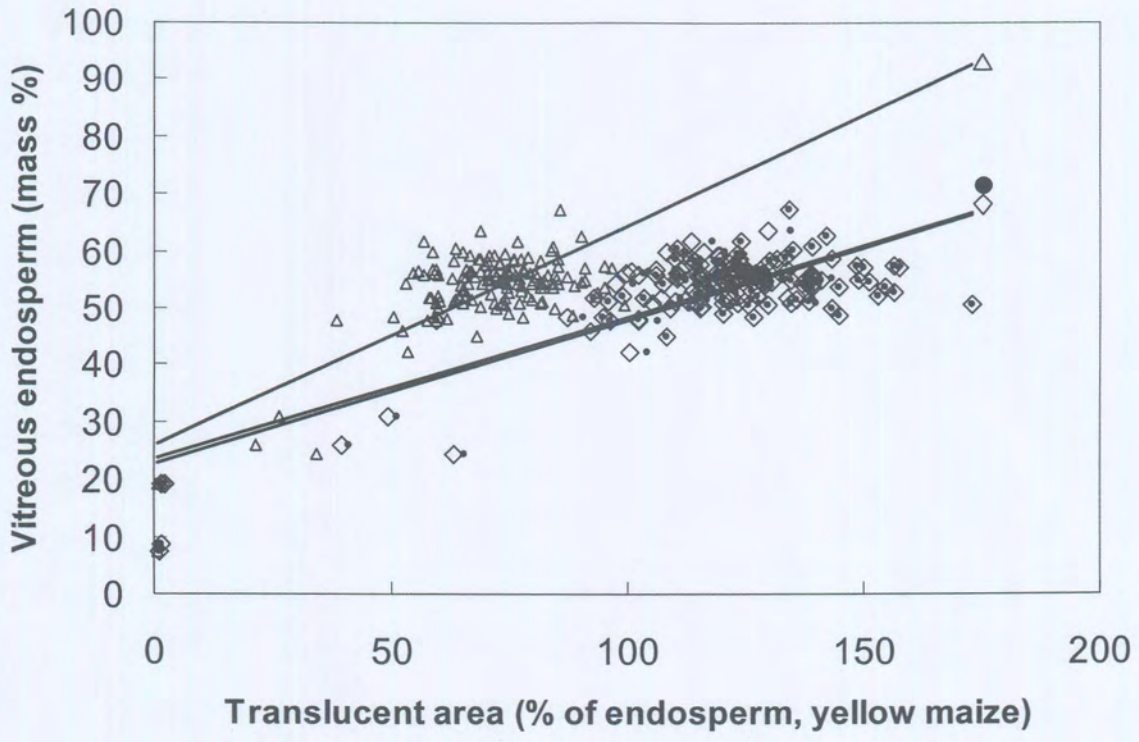

Figure 3.19 The effect of applying translucency correction factors (CFs) on the relationship between vitreous endosperm (mass %) as determined by hand dissection and the translucent area (% of endosperm) of yellow maize as determined using IA. "◊", before any CFs (y = 0.25x + 23, r = 0.80); "●", after thickness CF (y = 0.25x + 24, r = 0.79) and "△", after thickness and exposure CFs (y = 0.38x + 26, r = 0.76), n = 146. r-values did not differ significantly from each other (Fisher test). Slopes differed significantly from the horizontal

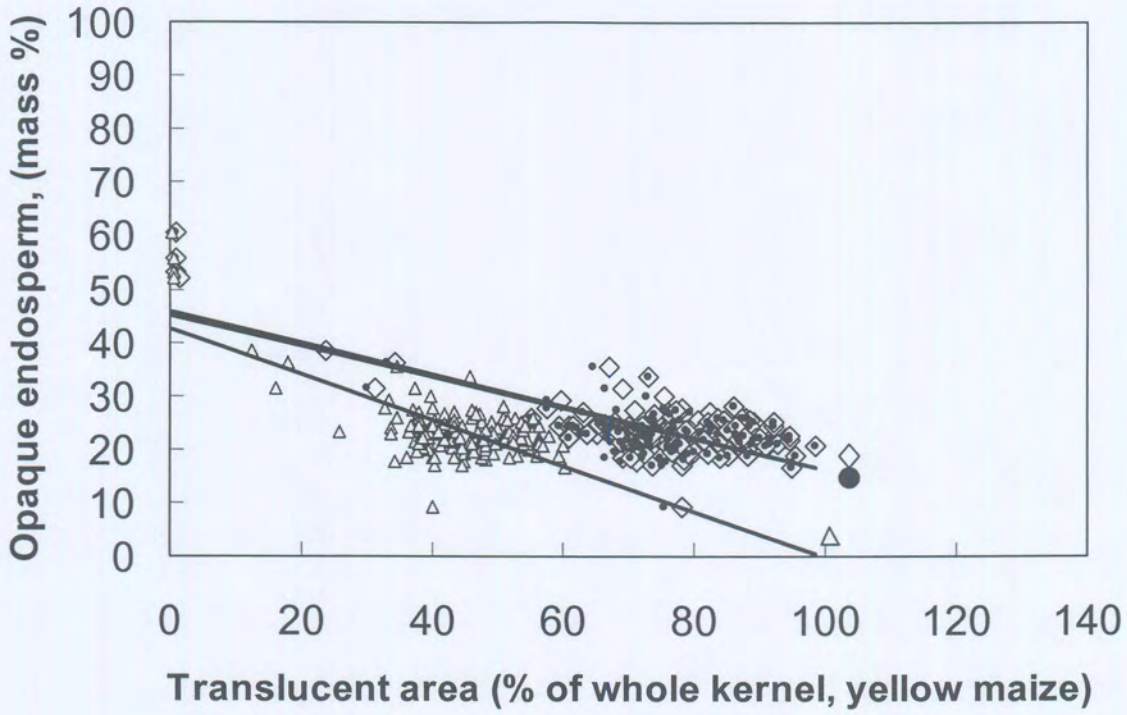

Figure 3.20 The effect of applying translucency correction factors (CFs) on the relationship between opaque endosperm (mass %) as determined by hand dissection and the translucent area (% of whole kernel) of yellow maize as determined using IA. "◊", before CFs (y = -0.30x + 46, r = -0.76); "●" after thickness CF (y = -0.29x + 45, r = -0.75) and "△", after thickness and exposure CFs (y = -0.43x + 43, r = -0.71), n = 146. r-values did not differ significantly from each other (Fisher test). Slopes differed significantly from the horizontal

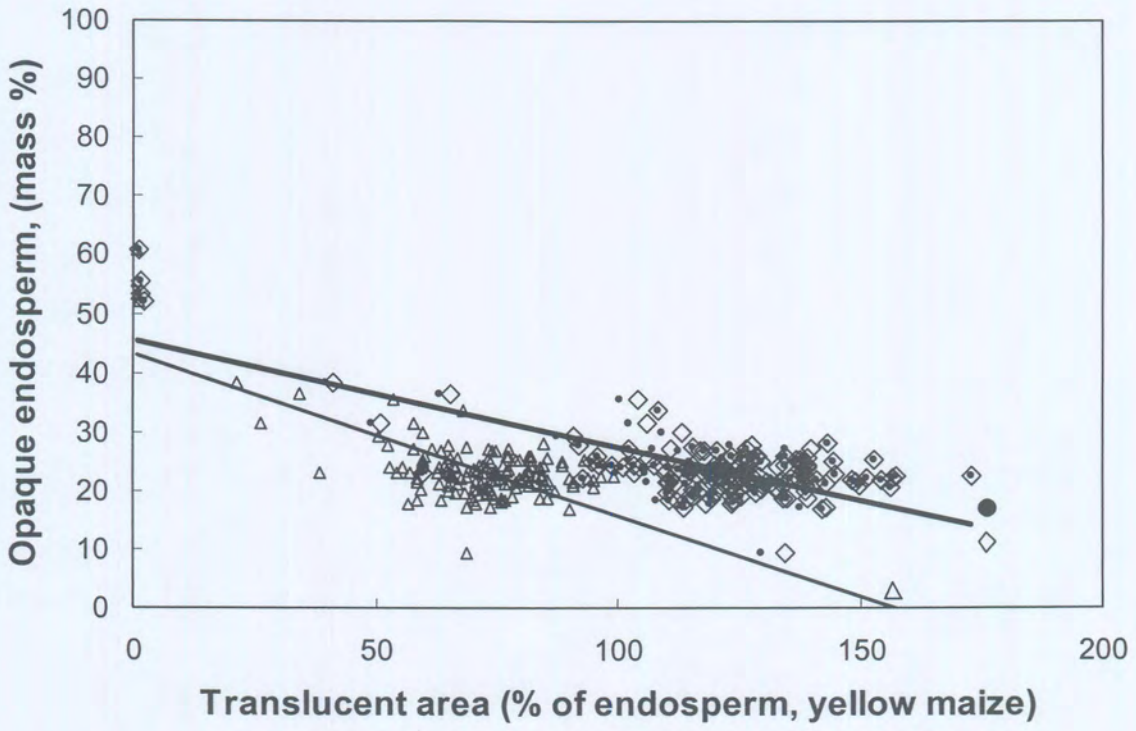

Figure 3.21 The effect of applying translucency correction factors (CFs) on the relationship between opaque endosperm (mass %) as determined by hand dissection and the translucent area (% of endosperm) of yellow maize as determined using IA. "◊", before CFs (y = -0.18x + 46, r = -0.76); "●", after thickness CF (y = -0.18x + 45, r = -0.75) and "△", after thickness and exposure CFs, (y = -0.28x + 43, r = -0.72), n = 146. r-values did not differ significantly from each other (Fisher test). Slopes differed significantly from the horizontal

Results of hand dissection measurements for white and yellow maize (Tables 3.9 and 3.10) as well as the results of the translucency measurements for white and yellow maize (Tables 3.11 and 3.12) showed statistically significant differences between cultivars. This indicated that the results were suitable for further analysis by linear regression. The three yellow cultivars (Table 3.12) had the same thickness.

Correlation coefficients for white maize (Table 3.13) increased after the application of the correction factors. Thickness correction had a bigger effect than exposure correction. For yellow maize (Table 3.14), thickness correction had no effect on the correlation coefficients, but corrections for exposure reduced the correlation coefficients. These trends occurred both for the vitreous and opaque endosperm results. For white maize, correlation coefficients improved significantly after thickness corrections. For white maize, these improvements were significant in most cases. As the yellow maize cultivars did not differ significantly in thickness (Table 3.12), the effect of thickness correction could not be demonstrated. Small differences in the actual thickness values did change the graphs very slightly, but not significantly. However, the yellow maize already had a highly significant and strong correlation before any corrections were made, as opposed to the white maize, which had a poor correlation before corrections. White maize cultivars also differed significantly in thickness.

Corrections for exposure had a bigger effect on the slopes of the linear regression lines than corrections for thickness (Figures 3.14 – 3.21). Slopes increased after corrections for exposure in all cases, both for white and yellow maize, while thickness corrections had only a limited effect with no changes in all cases except for the results in Figures 3.14 and 3.15 (relationship between translucent area as a percentage of the whole kernel and vitreous and opaque endosperm yields for white maize).

In all cases (Figures 3.14 – 3.21), the ranges of the translucency measurements were adjusted to realistic values after corrections for exposure.

Slopes differed significantly from the horizontal in all cases (t-test) which can be ascribed to the high number of observations.

3.3 DISCUSSION

It was clear from the beginning of the study that damaged kernels had a significant effect on the obtained image. As the amount of damaged kernels would vary among samples, they were excluded from the analysis.

As long as the selected circle size was smaller than the kernels, very few problems occurred with light escaping past the sides of the kernels. The method was significantly more convenient than having to embed each kernel in modeling clay and high-resolution images (Figure 3.3) could be obtained.

The camera used in the study was a T.V. standard high resolution digital camera using a 3CCD device for transforming the analogue light signals into digital form. The resolution total of 439300 pixels (764x575) is in the normal range for cameras used for machine vision imaging, although cameras with up to 1 gigabyte of pixels are available. Cameras used for machine vision imaging make use of a 3CCD device where the three primary colours are detected separately for more contrast (Leutron, 2004). The detection of kernels using a light table with a mask covering excess light was necessary because of the tendency of the light to reflect causing a significant loss of image contrast. The very bright background when viewing kernels without a mask caused a shadowing effect on the kernels that reduced the visibility of the internal structures of the kernels. Reflection of the light also caused features on the surface of the kernels to become visible which further reduced image clarity. Although "blooming" of image sensors, where very bright light contaminates adjacent darker pixels can be dealt with using the imaging and digital camera software, the corrections were not sufficient to eliminate the effect of shadowing and reflection, thereby making the use of the mask necessary.

Although only a preliminary test was done, humidity did not have any effect on the detected translucency. A humidity of 98% is high enough for mould growth on the kernels and was chosen as an extreme value (differing from the ambient relative

humidity) for detecting a potential effect. As no effect was observed, further analysis was discontinued.

The comparison of the triplicate readings using three maize kernels measured at four circle sizes is given in Table 3.4. The standard deviations show the detection differences between triplicate readings. These differences were caused by small changes in kernel position as kernels were placed by hand with the circles as closely as possible in the middle of each kernel as well as fluctuations in electricity and other machine-related "noise". These differences did not have a significant influence on differences between measurements at different circle sizes.

The results in Table 3.4 show an increase in detected translucent area for each increase in circle size. It was expected that the detected area would increase similarly for each kernel (a similar ratio). However, a statistically significant interaction was found between circle size and the individual kernels. This interaction is clear when comparing the increase in area sizes for each kernel. At a circle size of 17.2 mm^2, kernel 3 had the largest translucent area, but at a circle of 48.3 mm^2, kernel 3 had a translucent area similar in size to kernel 2 and both were smaller than kernel number 1. This indicates that there was more unexplained variability influencing the results and the two potential variables that were identified for further evaluation, were the ratio of the circle area to the surface area of the kernels and the thickness of the kernels. Highly significant differences occurred between the measurements taken at the four different circle settings (Table 3.4). The interaction was shown by the lower sensitivity of the measurements at the circles of 17.2 and 48.3 mm^2. At these two settings, two kernel translucent areas could not be distinguished from each other, while at settings number two and three (Table 3.4), all three kernel translucent areas could be distinguished from each other. The reason was that the ratio of the light area versus the kernel area had to be at an optimum. If the circle area was too small (circle of 17.2 mm^2), the kernel area was illuminated unevenly. If the circle area was too large, overexposure of the camera detector occurred with excessive light blurring the image. Kernels with small differences in translucency were detected without a significant difference. The circle of 29.6 mm^2

was sensitive enough to distinguish between the three different kernels and was chosen for future work.

Some maize kernels produced a single translucent area after detection, but most kernels produced two separate areas, due to the distribution of opaque endosperm in the hybrids. As the opaque endosperm tended to be concentrated around the germ in the maize kernels, it divided the translucent endosperm into two parts on either side of the germ. There are different combining methods for the two areas: connecting with a vector (thin straight line) or using the dilation and erosion feature, where pixels were added and subtracted according to set patterns (Leica QWin User Guide 1996). These methods were tested in order to select the best method with the least amount of changes to the measured area as illustrated in Figure 3.5. The different procedures had significant effects on the measured area. The vector method produced data that did not differ significantly from the unaltered measurement, but using the customary dilation and erosion method, produced significant differences in results. It was therefore decided to use the vector method of combining areas for future readings on multiple kernels. In the method of Felker and Paulis (1993) only single kernels were measured and the total area of gray was measured for the whole field. However, in this study, when multiple kernels were measured, the areas belonging to each kernel had to be combined in order to obtain the correct results. The combination method of choice will depend on the software available for the test. Software could also be developed to accommodate the unique measurements required in this work, for example allowing automatic combination of the two detected areas by the computer for each kernel using a programmed algorithm.

No significant differences occurred between repeat readings for measurements (Table 3.5). This indicated a negligible level of background noise from the images during detecting and that the measurements were highly repeatable. Kernel orientation (Table 3.5) had no significant effect on the detected translucent areas. This indicated that the circular shaped circles allowed for kernel areas to be detected

repeatably regardless of the kernel orientation. This will simplify a potential on-line detection process considerably.

As the maize kernels differed in size (variability which is characteristic of all biological material), it was suspected that using a fixed circle size for each measurement could have lead to additional variation in the translucent areas detected. Therefore, it was decided to compare measurements of the same kernels using a fixed circle versus modeling clay for excluding excess light.

A comparison of the two illumination methods is shown in Table 3.6. In the case of cultivars 1,3 and 4, the translucent areas detected at the fixed size circle and the modeling clay were identical, but for cultivar 2, the areas differed significantly. Cultivar 4 had virtually no translucent endosperm and was used as an extreme case reference point. The fact that the same area sizes could not be detected in all instances led to the conclusion that the ratio of the circle area to the kernel projected area had to be fixed mathematically in order to allow for similar amounts of light entering each kernel. The effect of the minimum gray detection threshold level on EX when the same three maize kernels were exposed to seven different circles is shown in Table 3.7. The gray detection levels were set at such a level that the same average detected translucent area was obtained for each set of three kernels. Analysis of variance showed that no differences existed between the translucencies of each set. LSD Paired comparison tests also confirmed that no significant differences existed between translucencies and the null-hypothesis as defined in section 3.1.5.1 was accepted (the alternative hypothesis that significant differences do exist was rejected). Following this result, a plot was made of gray threshold level versus EX to determine whether a significant relationship existed. A very strong highly significant linear relationship was found (Figure 3.6). This showed that it was possible to adjust for the effect of EX on gray level based on kernel size if the circle is fixed. However, a fitted line would have had to be developed with more maize kernels of various sizes and also two separate lines would have to be created for yellow and white maize.

From the results shown in Figure 3.6, it was concluded that using a constant circle size, the measured translucency of each kernel could be standardized, although maize kernels differ in size. This is an improvement to the Felker and Paulis (1993) IA method for quantifying maize translucency, as the relationship can be programmed into the computer software. In addition, any maize kernel of any size can be measured by simply placing it on a circle without the need for sample preparation such as modeling clay. In an on-line system, kernels could be moved over a fixed sized circle for quick detection. As the projected kernel area is also detected for every kernel during the assay, the size of the kernel can be taken into account if the size of the circle is known.

The effect of decreasing the thickness of kernels on the increase of the detected translucent area is shown in Figures 3.11 and 3.12. The relationships found were both linear and did not follow the curve of Lambert's law for the absorption of light (Sears, Zemansky and Young 1982). The probable main explanation for this is that maize kernels have a heterogeneous morphological structure (Watson 1987a). Additional scattering of light resulting from the different relative sizes and positions of the opaque endosperm and germ would have influenced the measurements. One of the biggest problems was to develop an effective method for measuring kernel thickness. In the developed method, kernels were sanded with abrasive paper to various thicknesses, then the translucent area was measured. It was not practical to remove the vitreous endosperm alone and sand it into disks of different thickness as it would not have resembled the effect of an intact kernel. Removal of the vitreous endosperm for the determination of absorption coefficients for comparison among cultivars, or endosperm colours in order to develop a better understanding of the factors influencing the absorbance of light by maize kernels could be considered. The scope of this work did not allow for further in-depth studies such as these.

The thickness measurement method developed in this work therefore only gave an indication of the thickness effect and will require some refining to confirm or adjust the thickness correction factor in future work. The effects of cultivar, relative size of germ, kernel shape and colour (except between white and yellow) were not

measured due to time constraints. It is strongly recommended to investigate these effects further in future as this might explain more variation in the translucency results. The slopes of the white and yellow maize kernel thickness effects were similar. This was not expected as the yellow colour had an affect on the general settings required to detect the total area of translucent endosperm. More levels of gray (from 44 to 255) for yellow kernels were measured than for white kernels (from 54 to 255). In spite of these differences, the reduction in detected translucent area will be the same with each mm increase in kernel thickness for both white maize and yellow maize. It is not known, however, how accurate the measurements were and the effect will have to be tested using the percentage translucency for the prediction of the yield of vitreous and opaque endosperm fraction in maize products (dry milled or hand dissected).

In order to accurately measure the mass percentage of opaque and vitreous endosperm, it was required to remove the opaque endosperm completely without damage to the kernels. To do this, it was necessary to soak the kernels for five days in water in order to have them soft enough to be cut open with a scalpel. Opaque endosperm was brushed out easily, except for some in the top end of the kernels. This could be scraped out easily using a sharp object such as a scalpel. Care had to be taken not to damage the germ during brushing, but apart from that, no other damage occurred to the kernels. As kernels would have fermented if kept in water at ambient temperature for five days, they were kept at refrigerated conditions. Vitreous and opaque endosperm could be easily distinguished in both white and yellow kernels. The strength of this improved maize dissection method is that it allows easy separation of the opaque endosperm from the rest of the maize kernel. It was found, however, that some opaque endosperm tended to stick to the vitreous endosperm in a thin layer which could not be brushed out without producing some damage to the germ. The germ becomes very soft during the soaking process and a few kernels had to be discarded after the whole germ came loose during the brushing. The biggest drawback of the procedure is that kernels had to be dried again afterwards which could lead to experimental errors if the drying is too severe.

Kernels must be dried slowly, preferably at temperatures below 50°C for the best results.

The summarized hand dissection data on the kernels are given in Tables 3.9 and 3.10. Vitreous endosperm percentage for white maize based on the whole kernel mass varied between 46.3% for the lowest amount and 63.7% for the highest amount. This range of 17.4% is narrower than ranges reported for other African maize cultivars (44.6%) (Louis-Alexandré, Mestres and Faure 1991) where weighed endosperm vitreousness was measured using hand dissection. The range for the yellow cultivars (Table 3.10) was narrow, between 49.9 and 56.4%. Both white and yellow cultivars have been bred and optimized in South Africa for various traits during the past few decades such as milling performance (personal communication, Randall, P.G., Director, P Cubed) and fungal resistance (personal communication, Esterhuyzen, A., Researcher, Monsanto). Therefore, the maize have become quite homogenous which would explain smaller variation in results.

The correlation coefficient r between % vitreous endosperm and % opaque endosperm as determined on the total of 245 kernels of the white maize was -0.946 ($P < 0.00001$), with $R^2 = 0.895$ (linear regression) showing that the dissection procedure was precise. For yellow maize $r = -0.935$ ($P < 0.00001$) and $R^2 = 0.874$. Vitreous and opaque endosperm was expected to be inversely proportional to each other as the amount of opaque endosperm lost during brushing will be reflected as a lower amount of vitreous endosperm retained. In order to reduce the effect of only subtracting the lost opaque endosperm mass from the total mass of the maize kernels, the vitreous endosperm was also dissected out and weighed after the pericarp and germ was removed. Unfortunately, the opaque endosperm cannot be dissected out as a stand-alone fraction as it disintegrates too easily. Opaque endosperm could only be measured as a mass difference before and after brushing. The results for the two sets for white and yellow maize were similar, showing that there was very small experimental error.

The same percentages of endosperm were obtained for fractions calculated on a dry basis and calculated on an "as is" moisture content. This is advantageous because no drying of fractions is necessary afterwards, provided that samples are weighed and dissected immediately after opaque endosperm removal and not allowed to dry out partially.

Mean values for IA on each maize hybrid are given in Tables 3.11 and 3.12. Variability of the translucent area measurements depended on the calculation method and the addition of the correction factors. The standard deviations decreased slightly or stayed the same after correcting for thickness (Trb), but decreased further in all instances after correcting for exposure percentage as well (Trc). This was observed both for the white and the yellow cultivars. This indicated that a portion of the variability in translucent area detected occurred as a result of faulty readings due to either over or under-exposure and in some cases due to thickness differences. The correction factors for exposure percentage can be programmed into the image analyser software for automatic correction at the detection stage. The calculation of the translucency as a percentage of the projected kernel area had the lowest standard deviation when calculated as a percentage of the total kernel area (Tr1, Tables 3.11 and 3.12). When the germ area was subtracted from the total kernel area before calculating the translucency percentage, the standard deviations increased slightly (Tr2). This increase is ascribed to the fact that the area of the germ had to be detected by indicating the area using a computer mouse marking the boundary between the germ and the endosperm. It could not be detected automatically because the contrast between the germ and the endosperm was not high enough for the computer to distinguish between the gray levels. This hand-detection method of the germ was prone to errors such as an inability to distinguish clearly where the germ boundary began and in many cases the germ area detected was too large. Pieces of pedicel (coming from the maize cob when the kernels were removed) often adhered to the tip cap and as the tip cap and germ areas were measured together, the pedicel pieces also increased the total area measured. The experimental error resulted in increased variability of the calculated translucent endosperm areas.

The individual samples showed similar standard deviations for vitreousness as those reported for vitreousness indexes calculated on sectioned kernels from other African maize cultivars (not South African cultivars) (Louis-Alexandré, Mestres and Faure 1991). However, as standard deviations for translucency indexes of individual samples have not been calculated previously for normal maize, the amount of variation cannot be compared. But it would also have been influenced by factors such as sample homogeneity and sample size. Translucency varied between 26.5% and 56% for Tr1 (a difference of 29.5%) and between 40.7% and 57.4% for Tr2 (a difference of 16.7%) for white maize. Translucency varied between 34.5 and 51.4% (Tr1) and 56.5 and 79.4% (Tr2) for yellow maize. The range of translucency was small when compared to translucency levels tested for high lysine maize (100% difference between lowest and highest levels) (Felker and Paulis 1993). In order to detect significant differences between samples within such a narrow range of translucency, sample size per cultivar was large (49 kernels) as compared to the 10 kernels used for the high lysine maize samples subjected to IA (Felker and Paulis 1993).

All correlation coefficients were significant at the 99.9% level ($P<0.001$) for white maize. For yellow maize, p was significant at < 0.00001. The high significance can be largely attributed to the high number of observations ($n = 245$ for white maize and $n = 146$ for yellow maize) and hence the resulting high number of degrees of freedom (Murdoch and Barnes 1973). There were significant negative relationships between opaque endosperm and translucency for both white and yellow maize. Before corrections, the difference between correlation coefficients of Translucency 1 and Translucency 2 for white maize was statistically significant (Table 3.13), but for yellow maize, the difference between Translucency 1 and Translucency 2 was not significant (Tables 3.14).

Larger standard deviations occurred in the measurements of Translucency 2 (Tables 3.11 and 3.12). This was possibly due to a small experimental error caused by adding the germ area by hand using the "draw" function on the image analyser. As the differences between the correlation coefficients for translucent area 1 and

translucent area 2 and vitreous endosperm was not statistically significant in any instance after all corrections, it can be concluded that both methods would be suitable for predicting percentage endosperm yield. To calculate the translucency 2 value involves excluding of the germ area by a manual step using the "draw" function. The manual step cannot be implemented during a quick in-line application. Translucency 1, however, can be calculated using the computer programme only.

Thickness correction increased the correlation coefficients of white maize significantly, indicating that the thickness of the kernels did account for a significant portion of the variation (Table 3.13). The thickness correction factor of 21.9 found in this work was smaller than the thickness correction factor of 36.5 for high lysine maize as calculated by Felker and Paulis, 1993. However, as the method for determination of the high lysine factor was not described in full by these authors, it is not possible to do a direct comparison. Thickness correction did not have a significant influence on gray measurements in high lysine maize (Felker and Paulis 1993) but it must be emphasized that translucency classes tested for high lysine maize varied between 0 and 100% in contrast to the variation of 29.5% in translucency of the results reported here for white maize. Although the thickness effect could not be demonstrated on yellow maize because the thicknesses of the three cultivars were the same, the thickness correction factor developed was exactly the same as the one for white maize and the factor will be used for further research.

After thickness correction (Tables 3.11 and 3.12), there were still many values indicating area percentages of more than 100%. This was the result of the effect of variable exposure percentages on the translucent area measurements due to variations in kernel sizes, but constant circle sizes (Figures 3.14 – 3.21). After corrections were made for exposure percentage in order to have a constant value for all detections, the slopes of the curves increased significantly, suggesting stronger relationships which accounted for more variation. The corrections for exposure also increased correlation coefficients slightly, but not significantly. Slope increase after corrections for exposure was consistently observed in all instances, both for white and yellow maize.

With the method developed in this study, 49 kernels could be analysed in five minutes when determining both Translucency 1 and Translucency 2 values. The sample size limitations were linked to the size of the camera lens as the speed of detection were the same for any number of kernels placed on the mask. However, as the kernels were placed manually on the mask and it was another time limiting factor. Larger sample sizes took longer to be placed on the mask, but a trained analyst could pack 49 kernels within one minute. Editing the germ area was more time consuming and it lengthened the total time for the analysis to 5 minutes for 49 kernels. The results of this study have shown that editing of the germ area is not necessary and it can be eliminated. Therefore, the time for analysing one sample using only Translucency 1 was reduced to 2 minutes. However, further research is needed in order to develop an automated system for placing the kernels on the mask. For in-line analyses, an automated sampling system will be needed to analyse representative samples taken from kernels moving past on a conveyor belt. With further research, a continuous system could be developed based on these observations.

3.4 CONCLUSIONS

A rapid non-destructive test has been developed for quantitatively measuring the translucency of large samples of maize kernels on a single kernel level using Image Analysis. No sample preparation is necessary (thereby for example removing the need to mount individual kernels in modeling clay) for these measurements and the sample size can be adjusted depending on the properties of the camera lens.

A correction factor to allow for constant illumination of kernels has been developed allowing the use of a single size light circle for illuminating maize kernels of varying size. As the projected area of kernels is measured during the assay, the correction factor can be programmed into the computer software to adjust illumination. The correction factor for exposure consistently increases the slope of linear regression lines fitted for correlating translucency and endosperm yield (vitreous as well as opaque) data for both white and yellow maize. It also eliminates translucency values of more than 100%, which are the result of overexposure in the case of small kernels.

A thickness correction factor, which has a significant effect on the strength of the relationships between translucency and vitreousness, has been developed for white dent maize. The thickness correction is significant within the narrow range of translucency (less than 30% difference between lowest and highest level) of the samples tested. A thickness correction factor was also developed for yellow maize, but its effect could not be demonstrated as the yellow cultivars measured did not differ in thickness. The yellow cultivars did, however, already give highly significant and strong correlation between translucency and vitreousness before any corrections as opposed to the white maize, which gave poor correlations before corrections.

The translucency of maize kernels is significantly correlated with vitreousness and opaqueness indexes determined by hand dissection. Relationships were developed for potentially predicting the yield of vitreous or opaque endosperm dry milling

products by using a translucency index allowing for possible future use as an analytical or quality control tool by maize millers.

In this work, kernels were placed over the illumination circle for transmitted light by hand. However, as kernel orientation had no effect on the detected translucent areas and good correlations were achieved without the need to measure the area of germ, the only requirements for accurate measurements are that the circles must be in the middle of the kernel and that the germ side must face the camera. At this stage in the development of the method, a trained analyst can measure up to 49 kernels in two minutes including packing the kernels on the mask if orientation is ignored.

During the research, it was found that if the light circles in the masks were not exactly in the middle of the kernels, results were not significantly influenced as long as no light escaped around the edges of the kernels. It was found that when kernels were placed ensuring that the light circles were covered completely, the circles tended to be in the middle of the kernels automatically possibly due to the selected size of the circles in relation to the average projected area size of the kernels.

The new method will allow for new insights into biological variation to be found within individual maize lines in terms of translucency as large numbers of kernels can be analysed in a quick and non-destructive manner.