Changes of gray level co-occurrence matrix (GLCM) texture and antioxidant level during freezing of pumpkin

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Abstract. GLCM (gray level co-occurrence matrix) textural features are rarely extracted from SEM (scanning electron microscopy) food images to study the effect of food processing on cellular microstructure and bioactive contents. Using the GLCM approach, the current study attempted to use textural information from pumpkin SEM images to obtain detailed cellular degradation caused by freezing treatment and to find their relationship with the antioxidant content. High dimensional textural features of the samples were obtained during image processing and subjected to multivariate analysis. The increase of antioxidant activity due to the freezing of pumpkin was used to create a vector factor to indicate the membership class of individual pumpkin samples. Following freezing, the flavonoid content of the pumpkins increased ranging from 15.27% to 70.39%. Freezing also caused the formation and increased of several volatile compounds detected by GCMS. The PCA (principal component analysis) results showed that two components were sufficient to explain the most variance of the dataset. Further supervised sPLS-DA (sparse partial least squares-discriminant analysis) indicated a clear separation of samples based on their antioxidant levels, suggesting an existing relationship between the texture and antioxidant. In conclusion, GLCM textural changes can be used accurately to examine the effect of freezing on the antioxidant of pumpkin.

1. Introduction
Pumpkins contain valuable natural antioxidants which easily deteriorate during post-harvest handling and processing. This necessitates a right preservation method of which freezing might be employed as low temperatures preserve heat-sensitive nutrients. In fact, an increase in antioxidants has been reported due to the freezing of plant food such as strawberry and spinach [1] [2]. The effect of freezing on food microstructure is commonly studied by SEM (scanning electron microscopy) imaging. Unfortunately, the results are much likely undermined by subjective judgment of the observer. Image analysis has gained great attraction for food science and industry as it helps to find the relationship between information contained in the image, structural modification, and the extractability of bioactive compounds [3].
Textural features are important image characteristic which can be used for identification purpose [4]. Several methods to obtain texture data are available, but the GLCM (gray level co-occurrence matrix) has been preferred as it provides the highest accuracy [5]. Indeed, the GLCM texture has been successfully employed to solve many classification problems [6]. In the GLCM method, texture features are extracted by statistical approaches from the co-occurrence matrix. In essence, an image GLCM is a matrix having row and column numbers equals to the number of gray levels of the image. A matrix element of \( P(i, j \mid d, \theta) \) represents the second-order statistical probability for changes between gray levels ‘i’ and ‘j’ at distance \( d \) and direction of angle \( \theta \) [7]. Fourteen textural features may be extracted from the matrix and the detailed explanation and mathematical formula used to calculate the features are given in the article [4]. Based on the high dimensionality of GLCM data, the multivariate analysis PCA is commonly used in image processing. PCA reduces the data dimension by finding a projection of the original set of vectors onto a lower-dimensional space with optimal mean-square distance [8]. In order to select factors which have the most influence on the component formed during PCA, an sPLS-DA may be applied [9].

Nowadays, textural data derived from microscopic images have been used to evaluate the effect of processing on the microstructure of foods. These include the effect of high hydrostatic pressure and pasteurization [3], the effect of cooking different kinds of pasta [10]. This would tremendously increase accuracy. There has been no information about the freezing effect on the pumpkin microstructure delineated by GLCM texture and its relation to the antioxidant activity. Therefore, this study was aimed to fill this gap of knowledge.

2. Materials and Methods

Mature, fresh and sound pumpkins were obtained from the traditional market in Malang. The slices of mesocarp of approximately 2x2x3 cm were obtained, frozen at -3°C, -8°C, and -18°C for different 4, 6, 8 hours. Fuzzy logic control was applied to obtain better control of freezing. Following thawing, the samples were prepared for SEM imaging at a magnification of 1000x. For flavonoid and antioxidant assays, 0.5 g oven-dried pumpkin powder was macerated in 95% ethanol for 24 hours and filtered using Whatman paper for analysis. The detailed preparation can be found in the previous report [11]. Antioxidant activities were measured by in vitro analysis using DPPH (1, 1-diphenyl-2-picrylhydrazyl) and ABTS (2,2-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid) methods. Total flavonoid contents were determined by the spectrophotometric method using quercetin as standard [12].

GLCM analysis was conducted for the SEM image of pumpkin samples. From within the SEM apparatus, the captured images were saved in the TIFF format. The images were then calibrated and converted to 8-bit depth. For each image, a region of interest (ROI) was assigned to represent more than 80% of the total area. The GLCM textures were then measured by texture analyzer v0.008 [13] plugin for image processing software ImageJ Ver 1.53a [14]. The algorithm used to process the images were the following: ROI of 700 x 700 um, pixel distance of 1 at angle direction of 0, 45, 90, 135. The resulting output is a vector of the following 11 textural features for each image: angular second moment (ASM), inverse difference moment (IDM), contrast, energy, entropy, homogeneity, variance, shade, prominence, inertia, and correlation. The unsupervised PCA (principal component analysis) and sPLS-DA (sparse partial least square - discriminant analysis) were conducted following the method for feature selection and multiple data integration [9]. The data input for the analysis consisted of a two-dimensional matrix representing individual samples and the average texture features of different angles respectively, and a factor vector of antioxidant activities which indicates the membership class of each sample. The class was formed based on the median of the factor. GLCM textures were subjected to correlation tests. The correlation and multivariate analyses were conducted within the R software environment for statistical computing, v3.6.2 [15] at \( p < 0.05 \).

GCMS analyses were performed on samples that have been prepared by the maceration method [16] with modification. Dried samples were extracted with hexane, then filtered for further analysis. The analyses were performed on pumpkin samples frozen at -18°C for 6 hours and untreated pumpkin for comparison. Shimadzu GCMS QP 2010 SE with ZB – AAA (10 mL x 0.25 mmLD (Phenomenex Inc.)
column was used with the following parameters: injection quantity 1 µL, chamber temperature 280°C, column oven temperature 60°C → (6°C/min) → 220°C, the pressure of 15kPa, injection mode split with a ratio of 127.5, gas carrier helium, the temperature of interface and ion source 280°C and 200°C respectively, solvent elution time 0.4 min with data sampling of 0.5 min to 7 min, scan mass range m/z 20-400 (3.33µ/sec), column and total flow 0.6 mL/min and 30 mL/min respectively, linear velocity 28.5 cm/sec, purge flow 3.0 mL/min, solvent cut time 2 min, and run time for 10 min.

3. Results and Discussion

3.1. GLCM textural features

![Graphs showing GLCM textural features](image)

*Treatment: A untreated, B frozen at -3°C4h, C -3°C6h, D -3°C8h, E -8°C4h, F -8°C6h, G -8°C8h, H -18°C4h, I -18°C6h, J -18°C8h*

**Figure 1.** Effect of freezing treatment on GLCM textural features of pumpkin.

The values of selected GLCM textural features of pumpkin samples for distance *d = 1* are presented in Figure 1. The graph shows that the freezing caused changes to the textures. ASM, IDM, contrast, and other GLCM textures were calculated based on values in the co-occurrence matrices. For each sample image, the matrix contained the numbers of how often different combination of pixel brightness values occurred in it. Essentially, the textures were determined by these brightness values or the gray levels of the images [17]. During the initiation of the image processing, conversion of sample images to 8-bit depth resulted in digital numbers representing the gray levels. ASM is a measure of image homogeneity where homogenous images have few dominant gray-tone transitions resulting in a higher value of ASM. Contrast represents local variation in an image, higher value means more variation while feature correlation represents gray-tone linear dependencies in the image [4]. Due to the calculation method,
some features are correlated [6]. Strong correlations were found between ASM and entropy (-0.93), homogeneity and entropy (-0.91), contrast and entropy (0.84).

3.2. Antioxidant increase

Freezing resulted in a substantial increase in flavonoid content ranging from 15.27% to 70.39% (Table 1). Because the flavonoid exhibits antioxidant property [18], both DPPH and ABTS measurements have also increased accordingly. Compared to others, which reported untreated pumpkin DPPH value of 40.82% [19], the baseline DPPH value in this recent study (32.33%) is slightly lower. The increases in antioxidant of frozen samples could be addressed to the release of the active compounds due to cellular disintegration. Slow freezing induces osmodehydration which destroys cell walls, vacuole, and cellular structure occurred during freezing and thawing [20]. An increase in antioxidants is a benefit gained from the processing as it can reduce the use of pumpkin as a material ingredient during food product development. Pumpkin antioxidants exhibit pancreatic protection and hypoglycaemic effects [21]. It can be used for food supplements or introduced in product formulation initiative for diabetics since the consumers have not perceived the antioxidant benefit as an important attribute for diabetic food product development [22].

Table 1. Increase in flavonoid and antioxidant activity of frozen pumpkin.

| Freezing temperature (°C) | Freezing time (h) | Flavonoid (mg/g DW) | DPPH (% inhibition, 500ppm) | ABTS (% inhibition, 500ppm) |
|---------------------------|------------------|---------------------|-----------------------------|----------------------------|
| Untreated                 |                  | 5.37±0.05           | 32.33±0.00                  | 26.19±0.11                 |
| -3                        | 4                | 6.19±0.02           | 37.34±0.13                  | 31.68±0.11                 |
| -3                        | 6                | 7.02±0.08           | 39.92±0.06                  | 35.59±0.13                 |
| -3                        | 8                | 6.51±0.04           | 38.19±0.00                  | 33.46±0.11                 |
| -8                        | 4                | 7.02±0.02           | 42.06±0.13                  | 38.73±0.00                 |
| -8                        | 6                | 5.81±0.01           | 34.44±0.13                  | 28.71±0.11                 |
| -8                        | 8                | 8.42±0.00           | 45.65±0.13                  | 46.88±0.00                 |
| -18                       | 4                | 7.47±0.01           | 43.20±0.13                  | 40.36±0.00                 |
| -18                       | 6                | 9.15±0.06           | 49.24±0.13                  | 54.90±0.21                 |
| -18                       | 8                | 8.96±0.01           | 48.68±0.13                  | 54.01±0.21                 |

3.3. Multivariate analysis

Based on the GLCM texture and antioxidant the multivariate was run to examine whether the major source of GLCM texture variance could be explained by antioxidant activity level. As shown in Figure 1, the GLCM textural features vary greatly, therefore during the data preparation a normalization with centering method was applied. The DPPH value, instead of ABTS, was chosen for inclusion in the multivariate analysis for the response vector considering that the method is accurate, rapid, simple, and less costly for antioxidant activity measurement in food [23]. Most importantly, DPPH and ABTS correlate positively (R²=0.972).

During the multivariate analysis, PCA was first to run to obtain an overview of textures data variation before building a model with sPLS-DA. The texture features included in the model were initially selected while keeping the distance at d = 1. As pointed out in the previous report, not all texture features may be useful to describe microstructural changes of food due to processing [3], therefore the potential features were selected by increasing the number of features between subsequent runs. The feature selected as starting points were based on the low statistical correlation they had, namely IDM,
homogeneity, and correlation. Another consideration when adding new features in the model was to optimize for the texture with a lower correlation.

The final result of the multivariate analysis is presented in Figure 2. As the figure shows, the most variance of the texture data can be explained by two principal components (PC1=87%, PC2=10%). It should be noted that as PCA is an unsupervised technique [9], the separation between individual samples could not be expected from this analysis. Rather, the most important information gained from the analysis is that the GLCM texture features may be reduced to one or two components while retaining much information as possible as shown on the projection graph.

Based on the PCA results, the sPLS-DA model was fitted to two components and the DPPH factor was included in the supervised mode. It can be seen from Figure 2 a clear separation of pumpkin samples based on their antioxidant level. The ellipses were set to show the strength of discrimination at a 95% confidence level. Samples frozen at higher temperatures, which are close to their initial point of freezing [11] exhibit different characteristics from those frozen at lower counterparts. The projection of the samples on the graph would be the evidence to draw a conclusion that GLCM textures are greatly affected by freezing treatments and they exhibit relation to antioxidant content in some ways. The existence of the relationship between GLCM textures and chemical properties of food, namely total starch and protein, has been reported earlier for pasta products [10].

**Figure 2.** Multivariate analysis of GLCM textures, samples were assigned based on freezing temperature (°C) and time (h). PCA scores (a), samples separation on sPLS-DA analysis (b).

| Angle | ASM (°C) | IDM (°C) | Entropy (°C) |
|-------|----------|----------|--------------|
| 0     | 0.00068  | 0.2120   | 7.5680       |
| 45    | 0.00089  | 0.1990   | 7.7680       |
| 90    | 0.00095  | 0.2120   | 7.5560       |
| 135   | 0.00088  | 0.1960   | 7.6910       |
| Average | 0.00092 | 0.2050 | 7.6200 |

**Note:** (a) Untreated sample, low DPPH, (b) Freezing at -3°C4h, low DPPH, (c) Freezing at -18°C6h, high DPPH

**Figure 3.** Comparison of textural features of frozen pumpkins.
The sPLS-DA result also indicated that the three most influencing features for the first component are IDM, ASM, and entropy with a loading value of 0.757, -0.403, -0.400 respectively. Thus, these textural features appear to be useful in determining the effect of freezing on the cellular microstructure of frozen pumpkins. IDM and entropy have been reported also as important features in examining the microstructure of red sweet pepper due to high hydrostatic pressure and pasteurization [3]. The visualization of texture characteristics of the sample images along with their most important features to the first principal component are depicted in Figure 3. It can be seen that treated samples exhibit lower homogeneity (ASM) compared to untreated samples. Images with less dominant-gray tone transition is considered more homogeneous [4]. The frozen samples entropies are higher than that of the untreated, indicating more complex variabilities of the textures [17]. These indicate that frozen pumpkin surface are more irregular to higher extent compared to untreated sample.

GCMS analysis was run to confirm changes of volatile compounds related to microstructural decomposition reflected by the GLCM texture. For this purpose, two samples supposedly to have different texture characteristics were selected. One was untreated and the other was the frozen samples with the highest antioxidant content, assuming underwent most cellular degradation. The GCMS result showed that freezing caused the formation of new volatile compounds, namely α-pinene, limonene, linalool, isovaleraldehyde, β-cyclocitral, β-ionone. The results also indicated an increase in volatile compounds Table 2. Limonene and α-pinene had the highest increase. This could be addressed to the effect of cellular decomposition due to freezing and subsequent thawing which may cause the release of the compounds. As volatile compounds of pumpkin serve important factors in determining pumpkin species [24], the changes during processing such as freezing may have influenced the result. It is interesting to note that the volatile increase coexisted with antioxidant change. This would necessitate further confirmatory work to conduct as related data has been currently scanty.

| Retention Time | Compound | Formula | Mass | Curve Area Increase |
|---------------|----------|---------|------|---------------------|
| 0.96          | Acetaldehyde | C\textsubscript{2}H\textsubscript{4}O | 44,0262 | 11,445,790 |
| 1.04          | Propanal  | C\textsubscript{3}H\textsubscript{6}O | 58,0419 | 12,653,370 |
| 1.05          | 2-methylpropanal | C\textsubscript{4}H\textsubscript{8}O | 72,0575 | 17,749,090 |
| 1.16          | Ethyl acetate | C\textsubscript{4}H\textsubscript{8}O\textsubscript{2} | 88,0524 | 21,649,090 |
| 1.17          | 2,3-butanedione | C\textsubscript{4}H\textsubscript{8}O | 86,0368 | 14,034,520 |
| 1.22          | Propyl acetate | C\textsubscript{5}H\textsubscript{10}O\textsubscript{2} | 102,0681 | 18,736,570 |
| 1.24          | Ethyl butanoate | C\textsubscript{6}H\textsubscript{12}O\textsubscript{2} | 116,0837 | 17,938,090 |
| 1.26          | 2-ethylfuran | C\textsubscript{6}H\textsubscript{12}O | 102,0681 | 18,736,570 |
| 1.42          | Ethyl-2-methylbutanoate | C\textsubscript{7}H\textsubscript{14}O\textsubscript{2} | 130,0994 | 20,545,770 |
| 1.48          | α-pinene | C\textsubscript{10}H\textsubscript{16} | 136,1252 | 67,777,130 |
| 1.49          | Limonene | C\textsubscript{10}H\textsubscript{16} | 136,1252 | 57,549,590 |
| 1.54          | 2-pentylfuran | C\textsubscript{9}H\textsubscript{18}O | 138,1045 | 18,643,580 |
| 2.11          | Ethyl hexanoate | C\textsubscript{8}H\textsubscript{16}O\textsubscript{2} | 144,1150 | 12,610,840 |
| 2.60          | Butyl-2-methylbutanoate | C\textsubscript{8}H\textsubscript{16}O\textsubscript{2} | 158,1307 | 11,859,090 |
| 5.21          | Hexyl hexanoate | C\textsubscript{10}H\textsubscript{20}O\textsubscript{2} | 172,1463 | 12,641,870 |

4. Conclusions
Freezing substantially corresponds to the changes of pumpkin GLCM textures which likely represent the cells disruption caused by the treatment. The disruption could be the responsible factor for the release of the antioxidant and volatile compounds. Multivariate analyses of PCA and sPLS-DA reveal that samples with high antioxidants exhibit different GLCM textures with those of low counterparts. The important textural features to examine the effect of freezing on cellular disruption and antioxidants include IDM, ASM, and entropy. In practice, it is highly recommended to take advantage of GLCM
analysis to accurately examine SEM images data to provide objective information in evaluating the particular effect of food processing.

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