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# ACCURATE QUANTIFICATION OF FUNGAL GROWTH IN BREAD BY USING SPECTRAL ANALYSIS

**Morsy, Noha. El[1]; S. M. Mokhtar [2] and Kh. M. Youssef [2]**

**1-Suez Canal University, Faculty of Agriculture, Food Science and Technology Department, Home Economics Branch**

**2-Suez Canal University, Faculty of Agriculture, Food Science and Technology Department**

## ABSTRACT

Traditional methods for detection and enumeration of microbial growth in food stuff are very time consuming, destructive, invasive, complex, expensive and risky especially in case of pathogenic microbes. Therefore, the experimental work comprehensively detailed in this study was carried with the aim of non-destructive detection and quantification of fungal growth in bread using spectral analysis as one of the most promising techniques. For a period of seven consecutive days, spectral images in the near infrared (NIR) range were acquired for freshly-backed bread samples. Concurrently, the corresponding mould growth was monitored and assessed with the standard plating methods. Spectral data extracted from the images of bread samples and their reference mould counts during the storage period were modelled using multivariate statistical models. The principal component analysis (PCA) indicated that bread samples at the first four days consistently had similar spectral fingerprint and projected at the same location in the principal component plot. Starting from the fifth day, bread samples exhibited extraordinary spectral behaviour. Moreover, results demonstrated good prediction of mould counts in calibration and validation sets of bread samples ($R_C^2$ = 0.97 and $R_P^2$ = 0.94). The results presented in this work revealed that the biochemical fingerprints during fungal invasion conveyed by NIR spectral images in combination with the appropriate multivariate analysis strategy have significant potential for rapid assessment of bread spoilage.

**Keywords:** Bread, safety, spectral analysis, Fungi, moulds

## INTRODUCTION

The demand for high quality and safety in food production calls for high standards in quality and process control, which in turn requires sensitive and rapid analytical technologies, sampling methods and data analyses. Perhaps no other food staple comes in such infinite varieties as bakery products. However, microbial spoilage is a major problem in bakery products since it can induce nutritional losses, off-flavours and formation of mycotoxins or potentially allergenic spores. Bread is prone to a rapid microbial spoilage, particularly mould growth, due to post-baking contamination during cooling, slicing and wrapping, which greatly limits its shelf life (Liu *et al.*, 2014). This situation can lead to an organoleptic deterioration of already marketed bakery products, which indeed threatens consumers' confidence and, therefore, results in huge economical losses. Therefore, housewives usually keen about the ideal methods for keeping bread fresh and edible as long as possible. They often want to learn how to store bread correctly to extend its shelf and prevent mould growth. This is the reason for a growing need to find a method

to conveniently assess the degree of fungal growth in bakery products at a very early stage and before it becomes visible (Needham and Magan 2002).

Mould spoilage accounts for 1-5% of product losses depending on the season, type of product being produced and the method of processing (Guynot *et al.*, 2002). In a humid atmosphere, however, and especially if the bread is wrapped, moulds will grow rapidly. This is true especially if the bread is wrapped hot from the oven so that droplets of water condense on the inside surface of the wrapped. When bread is cut, the inner, more susceptible surface is exposed to mould infection. The bakery product's water activity ($a_w$) is the most important factor affecting the type and rate of spoilage (Membré *et al.*, 1999). Commercially produced and properly handled bread generally lacks sufficient amounts of moisture to allow growth of any micro-organisms except moulds. As normal cooking temperatures destroy fungal spores, post-process contamination from airborne spores and contact with contaminated surfaces must be prevented.

Traditional cultural detection of most micro-organisms requires growth of the organism on selective media, which can take a number of days from isolation to identification. These methods are sensitive, inexpensive and give qualitative information on the number and the nature of the micro-organisms present in a food sample. However, conventional methods require several days to produce results because they rely on the ability of micro-organisms to multiply to visible colonies. Moreover, culture medium preparation, inoculation of plates, colony counting and biochemical characterisation make these methods labour intensive (De Boer and Beumer, 1999). Traditional methods are of limited value especially for the analysis of perishable foods since the foods are sold and eaten before the results of the tests are known. Rapid methods can reduce the time taken to achieve results from days to a few hours or even minutes. Numerous instrumental methods have been established in the course of the twentieth century and are developing further, together with data analysis techniques, for such purposes. Several investigations have focused on the optical technology exploitation as a rapid and non-destructive method to measure the quality and safety of food and agricultural products (Lawrence *et al.*, 2003; Park *et al.*, 2007 and Farkas and Dalmadi, 2009). Among them, NIR spectroscopic methods and chemical sensor arrays called electronic noses show particular promise for rapid, non-destructive, non-invasive and cost-effective ways for assessing changes and enhancing control during processing and storage of foods. Their key advantages as analytical tools are 1) their relatively high speed of analysis, 2) the lack of a need to carry out complex sample preparation or processing, 3) their relatively low cost, and 4) their suitability for on-line monitoring or quality control.

Screening for micro-organisms by spectral analyses is still in the beginning stages (Davies *et al.*, 1987). For instance, Sørensen and Jepson (1997) used NIR spectroscopy for detection of *Clostridium tyrobutyricum* in cheese. These works demonstrated the promise of NIR spectral analysis for microbial qualitative analysis. In addition, Lin *et al.* (2004) also used NIR for the quantitative analysis for determination of microbial contamination in chicken meat, detecting initial stages of mobilization processes during

germination of cereal grains (Farkas and Dalmadi, 2009)**.** Spectral imaging technology is a new rapidly emerging technique which enables contactless monitoring of attributes in complex systems. Recent studies have shown that spectral imaging employed in the visible and NIR range, in combination with multivariate analysis was able to correlate with the bacterial spoilage process in meat samples (Barbin *et al.*, 2013 and Feng *et al.*, 2013). The spectral technique has been applied for detecting fungal contamination and rust detection in grain (Asher *et al.* 1982), to assess mould contamination of tomato puree (Davies *et al.* 1987), evaluation of bacterial contamination in shredded cabbage (Suthiluk *et al.* 2008) and freshness assessment of Arabic flat bread (AFB) during the storage. However, in order to develop a practical and stable system, more sophisticated work must be done.

The main objective of the study was to implement spectral characterisation in tandem with appropriate multivariate analyses for early detection and quantification of mould growth contamination in bread slices before the presence of visible spoilage.

## MATERIAL AND METHODS

Freshly baked bread used in this study was purchased at a local supermarket sold as slices of 1cm thick in sealed plastic bags. The bread was transported to the laboratory and kept in a humidity controlled chamber adjusted at humidity of 70 % and a temperature of 20ºC throughout a storage period of 7 days.

**Imaging spectroscopy system**

Near-infrared (NIR) spectral images were acquired in the reflectance mode using a pushbroom imaging spectroscopy system. The system composed of spectrograph (ImSpector, N17E, Spectral Imaging Ltd, Finland), a charged couple device (CCD) camera with C-mount lens (Xeva 992, Xenics Infrared Solutions, Belgium), illumination unit consisting of two tungsten-halogen lamps (V-light, Lowel Light Inc, USA), a conveyor (MSA15R-N, AMT-Linearways, SuperSlides and Bushes Corp., India), a data acquisition software (SpectralCube, Spectral Imaging Ltd., Finland) and a computer (Feng *et al.*, 2013).

**Image acquisition and pre-processing**

The system presented in this paper produces images with dimensions of $x \times y \times \lambda$, which can be interpreted as a number of single sub-images ($\lambda$) each with $x \times y$ pixels. The conveying belt was driven by a stepping motor with a user-defined speed of 2.7cm $s^{-1}$ to move the bread samples in the field of view of the camera. The spectrograph and camera assembly collected spectral images in the near infrared spectral range of 897-1753 nm with a spectral resolution of about 3.34 nm between the contiguous bands producing a total of 256 bands. To avoid noisy bands, images were resized to the spectral range of 910 nm to 1700 nm with only 237 bands.

A white image (100% reflectance) was acquired from a white reference Teflon tile, and a dark image (0% reflectance) was obtained with the light source off and the camera lens completely covered with its opaque cap. Black (D) and white (W) images were used to correct the raw images

($R_0$) from the dark current effect of the camera and obtain the relative reflectance image (R) from each raw image using the following equation:

$$R = \frac{R_0 - D}{W - D} \qquad (1)$$

In addition, an ordinary colour image was also acquired for each bread slice using an ordinary colour camera on the same surface of the sample where the spectral images were obtained and then converted into CIE L*a*b* colour coordinates to investigate the influence of colour changes during the microbial spoilage of bread (Barbin *et al.*, 2013).

**Microbiological analysis**

Microbiological analysis of the bread slices were performed every day for seven congestive days. At the first day (Day 1), the slices were taken immediately before storage and then imaged and directly analysed. On each sampling day, eight randomly selected samples were removed from the controlled chamber and one spectral image and one digital image were acquired for each sample. After imaging, samples were examined for moulds per weight of bread by classical microbiological plating methods.

Directly after imaging, the sample was placed inside a stomacher bag (Seward Medical, London, UK) and weighed. Moulds determined by pour plate method using Malt Extract Agar (Oxoid CM0059; Unipath, Basingstoke, UK) and the pH for the medium was adjust to 3.5 by adding 1 ml of lactic acid 10% (Oxoid SR0021) to each 100 ml of medium at 50°C. Then the plates were poured with agar medium and allowed to solidify before adding the diluents. Each sample was then diluted decimally in peptone water and homogenised for one minute in the stomacher (Circulator 400; Seward Medical, London, UK). The resulting bread homogenate was serially diluted (1:10, $1:10^2$, $1:10^3$ etc.) in sterile peptone saline solution and appropriate portions (0.1 ml) of serial dilutions were spread on the surface of the solidified agar medium in the Petri dishes. Dishes were incubated at 25°C ± 2.5°C for 3 to 5 days for the determination of the moulds growth. All analyses were carried out according to standard plate-count method (ISO: 16212, 2008) and the moulds counts were expressed as cell-forming units (cfu) per gram of bread sample and then transformed into log values ($\log_{10}$ cfu $g^{-1}$).

**Image analysis**

Image segmentation is the first critical stage in image processing, as extracted data are highly dependent on the precision of this operation. Images were segmented with the objective of identifying only the bread sample as the region of interest (ROI) to be analyzed and separation from the background or other undesired region. All images were processed and analyzed individually according to the following routine. First, two sub-images (at two different bands) with a wide variation in reflectance were selected from the spectrum. Afterward, the two sub-images from the respective bands were arithmetically subtracted from each other followed by a simple thresholding at a constant value of 0.2. In the next step, a sequence of morphological operations of erosion was applied to exclude any pixel not related to the sample. This step produced a segmented image of the sample with the isolated flat part of the bread slice as the main ROI to be used for

extracting spectral data from each sample. The sequential procedure for image segmentation is explained in details in Barbin *et al.* (2013)..

**Spectral data analysis**

Spectroscopic data always contain a large amount of highly correlated data from neighbouring wavelengths. Multivariate data analytical tools such as principal component analysis (PCA) and partial least squares (PLS) regression have proven to be powerful methods for mathematical extraction of the dominant latent data structures of such collinear spectral data (Christensen *et al.*, 2006). Therefore, the initial diagnosis of the spectral behaviour of the bread samples through the whole storage period was preliminary carried out using principal component analysis (PCA) to examine any possible grouping of samples and to obtain an overview of systematic spectral variations among bread samples. The first few PCs retain most of the variation presented in the original spectral data. Accordingly, samples projected in the same location around a principal component seem to posses similar spectral features.

Also, prediction models were developed using partial least squares (PLS) regression for distinguishing bread samples stored at different periods and predicting the mould counts. The PLS model (PLS Model 1) was then built between the spectral data matrix (X) at the full wavelength and the variable (Y) containing the real measured counts of mould in bread samples. Moreover, spectral data at the most important wavelengths selected from the PLS regression coefficient as described by Barbin *et al.* (2013) were then used for the same classification and prediction purpose (PLS Model 2). Models built using calibration data set were optimized by using a separate validation set. In order to perform an external validation, all samples were divided into a calibration set (2/3 of the samples = 38 bread slices) and a validation set (1/3 of the samples = 18 bread slices). The resulting models were then evaluated by determination coefficients ($R_C^2$ and $R_P^2$), standard error (SEC and SEP) in the calibration and validation, respectively. All data transformation and subsequent multivariate analyses were performed in Unscrambler software (CAMO, version 9.7).

## RESULTS AND DISCUSSIONS

**Spectral characteristics of bread samples**

The average near infrared (NIR) reflectance spectra extracted from bread samples examined at the seven congestive days were converted to absorbance values (Absorbance = log(1/Reflectance)) and plotted against wavelengths as shown in Figure (1). Generally, the peaks observed in the NIR region are related to overtones and combinations of vibrations of C–H, N–H, O–H and S–H functional groups. The peaks at 974 nm and 1440 nm were very obvious in the bread samples and it is ascribed to water absorption bands related to O–H stretching second overtones (Barlocco *et al.* 2006 and Prieto *et al.* 2006), whereas a second peak at 1211 nm was due to the fat absorption related to C–H stretching second overtone (Andrés *et al.* 2008; Cozzolino and Murray 2004). Generally, the spectra for the bread samples till the fourth day appeared to be remarkably similar in their spectral attitudes,

but only differed in the magnitude of absorbance. Starting from the 5th day, it was also evident that the absorbance values for bread samples were entirely different from the normally fresh bread specially in the NIR range of 1200-1700 nm. Bread samples at the 6th and 7th day had higher absorbance in the spectral range of 910-1200 nm and lower absorbance in the spectral range of 1200-1700 nm.

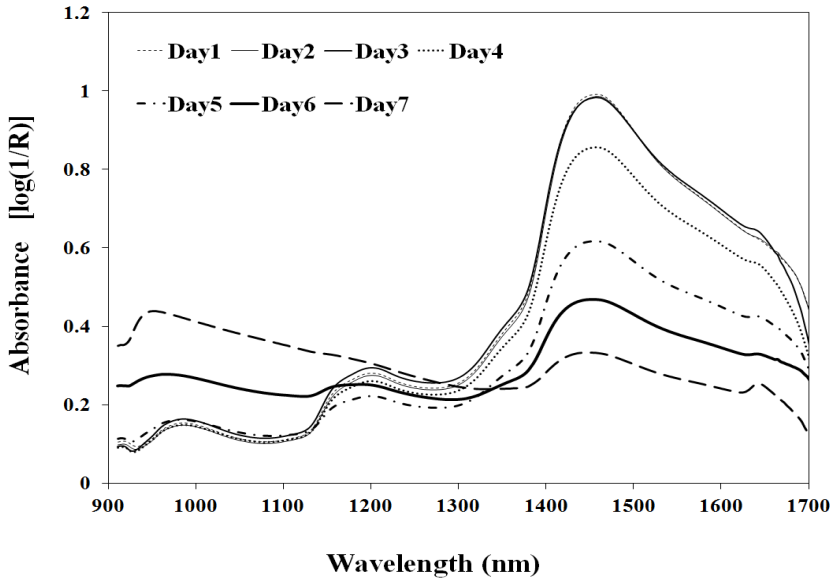


**Figure (1): Spectral characteristics of bread samples during storage period.**

Moreover, with prolonged storage, the absorbance valley at 1080 nm and the peak at 1200 nm were wholly disappeared and a new absorbance peak at 1640 nm started to exist. This feature is obviously related to the profound effect of mould appeared in the bread samples at these particular periods. Generally speaking, the different spectral attributes of bread samples are associated with different physicochemical characteristics of the tested samples. The result suggested that discrimination among bread samples stored at different storage periods is possible based on their spectral characteristics.

The same conclusion could be figured out from the score plot resulting from the principal component analysis (PCA) of the bread samples as shown in Figure (2). The first two principle components explained 99.47% of the variation among tested samples where the first PC explained 93.09% and the second PC explained 6.38% of the variation. The score plot of the PCA indicated that bread samples at the first four days consistently had similar spectral fingerprints and projected at the same location in the principal component plot. Starting from the 5th day, the bread samples exhibited extraordinary spectral behaviour.

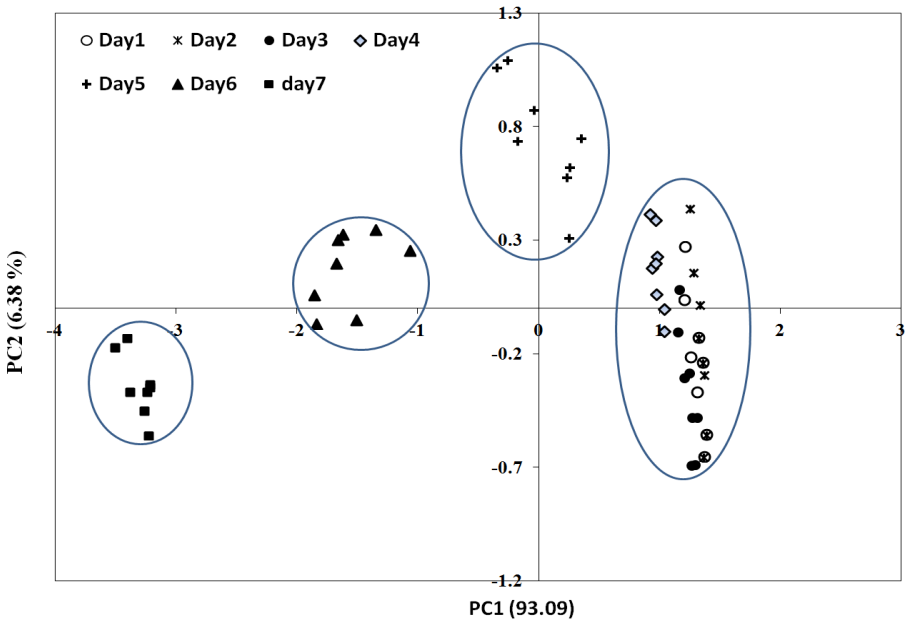


**Figure (2). Principal component analysis of spectral data extracted from bread samples at different storage intervals.**

It could be concluded that storing bread for four days under this condition could be eaten safely. However, storing bread beyond four days lead to very sever changes in the bread characteristics due to mould growth that alters bread properties and eating ability. This finding is in agreement with those reported by Latif *et al.* (2005) who noticed the spoilage of bread samples after four days of storage under similar conditions. That is due to the fact that during packaging of bread slices not all oxygen present in the product is eliminated and only low level of residual oxygen (1-2%) are sufficient to allow mould growth that rapidly augmented in the humid atmosphere (Stoops and Van Campenhout, 2012). Also, sliced, wrapped bread is more at risk, because the moist, cut surfaces are an ideal substrate for moulds to grow and the packaging prevents the moisture loss (Cauvain and Young 2007).

In other words, the PCA plot revealed that it could be possible to differentiate between safe and unsafe bread samples regarding mould growth using their spectral fingerprints. Furthermore, by inspecting the colour images acquired for bread samples as shown in Figure (3), we can easily reach to the same conclusion because the visual appearance of bread samples till the fourth day was identically similar. Meanwhile, starting from the 5$^{th}$ day the mould started to grow and finally rapidly distributed in every spot as shown at the 7$^{th}$ day of the storage indicating an unsafe state of these samples after prolonged period of storage under this condition.

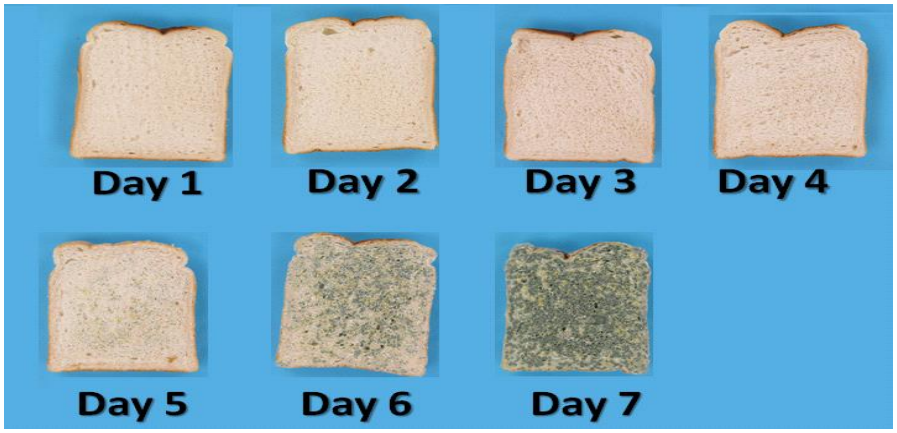


**Figure (3): Colour images of the examined bread samples acquired at different storage periods.**

**Performance of Partial least squares (PLS) models**

Since the count of moulds increases with increasing storage time, it is essential to develop a multivariate model for predicting the corresponding counts of mould during the storage period. The performance of the developed PLS regression models in predicting and precisely quantifying the number of moulds in bread samples are tabulated in Table (1). The results indicated that the developed PLS models were very accurate in predicting mould count with determination coefficient of 0.97 and 0.94 with standard error of 0.49 and 0.65 in the calibration and validation data sets, which indicates the robustness of these models. Similarly, the important wavelengths (924, 1058, 1178, 1295, 1366, 1400, 1497, 1611 and 1648 nm) selected from the weighted regression coefficients of the PLS regression model were used in building a new optimized model in predicting mould counts yielding determination coefficients of 0.94 and 0.95 with standard errors of 0.7 and 0.64 in the calibration and validation data sets, respectively.

**Table (1): Performance of partial least squares models in quantifying mould growth using the full spectral range (PLS Model 1) and the feature-related wavelengths (PLS Model 2).**

| Model | No. of Wavelengths | $R^2_C$ | SEC | $R^2_p$ | SEP |
|---|---|---|---|---|---|
| PLS Model 1 | 237 | 0.97 | 0.49 | 0.94 | 0.65 |
| PLS Model 2 | 9 | 0.94 | 0.7 | 0.95 | 0.64 |

The relationships between the actual mould counts estimated by the traditional plating method and those resulting from PLS regression models using the full spectral range and the important wavelengths (924, 1058, 1178, 1295, 1366, 1400, 1497, 1611 and 1648 nm) are illustrated in Figure (4) and Figure (5), respectively. It is very evident to figure out that both models were very accurate in predicting mould counts using the spectral background of the bread samples

**(a)**

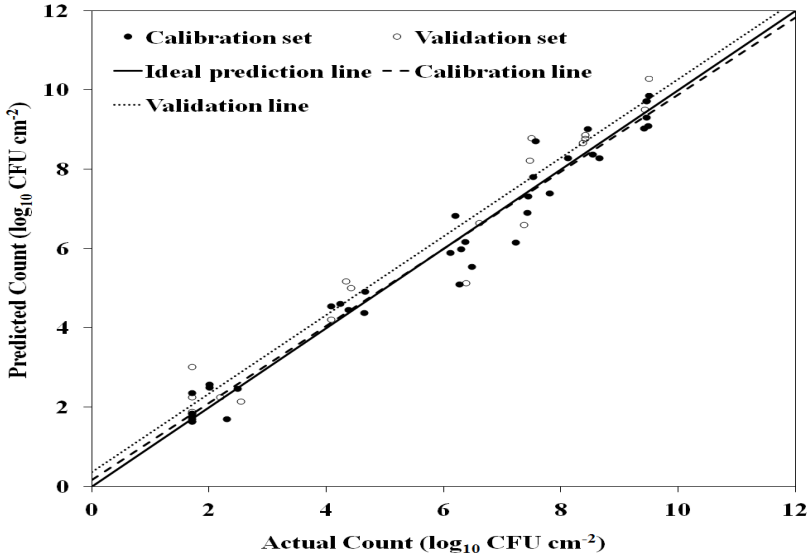


**Figure (4). Experimentally observed mould counts assessed by standard plat method against the predicted mould counts by the PLS models using the full spectral range.**

**(b)**

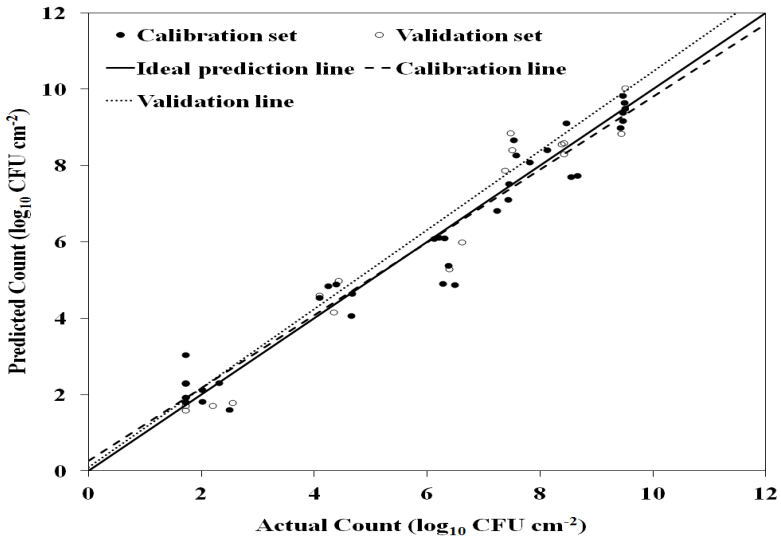


**Figure (5). Experimentally observed mould counts assessed by standard plat method against the predicted mould counts by the PLS models using the important wavelengths.**

**Conclusion**

The present study was initiated to employ a spectral analysis technique for non-destructive detection and quantification of fungal growth in bread as one of the most promising techniques. The spectral data extracted from bread slices were analysed by PCA and PLS multivariate analyses. The results revealed that storing bread under humid condition for four days could be eaten without hazard of mould growth. However, storing bread beyond four days lead to very sever changes in the bread characteristics due to mould growth that alters bread properties and eating ability. Therefore, it could be possible to predict mould growth in bread samples using their spectral fingerprints. The PLS models developed from the full spectral range (237 wavelengths) and the most important wavelengths (924, 1058, 1178, 1295, 1366, 1400, 1497, 1611 and 1648 nm) were very efficient in predicting mould counts in bread samples. The results presented in this work revealed that the biochemical fingerprints during fungal invasion conveyed by NIR spectral images in combination with the appropriate multivariate analysis strategy have significant potential for rapid assessment of bread spoilage. The non-destructive mode of analysis is of fundamental scientific importance, because it enhances the exploratory dimension to the measurements, allowing for more complex relationships such as the effects of fungal growth in the examined food samples. By this way, it is possible to properly estimate the mould growth in food stuff and avoid the costly and tedious methods even without prior and specific sample preparation. To our knowledge, this is the first study to implement spectral imaging technique in microbial detection in bread and the work presented here represents a primary study and requires further research endeavours. More research is needed to involve more samples as well as different bread qualities, processing regimes and storage conditions to ensure the accuracy and robustness of the developed models.

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## التقدير الكمى الدقيق للنمو الفطرى فى الخبز بإستخدام التحليل الطيفى

**نها السيد مرسى[١]، سيد مختار[٢] و خالد يوسف[٢]**

**١- جامعة قناة السويس - كلية الزراعة - قسم تكنولجيا وعلوم الأغذية - شعبة الإقتصاد المنزلى**

**٢-جامعة قناة السويس - كلية الزراعة - قسم تكنولجيا وعلوم الأغذية**

تتطلب الطرق التقليدية لإكتشاف وتقدير معظم الكائنات الحية الدقيقة إنمائها على بيئة إنتقائية خاصة لعدة أيام لعزلها وتعريفها. وبالرغم من أن هذه الطرق حساسة وغير مكلفة وتعطى نتائج دقيقة ومعلومات كمية لعدد وطبيعة الكائنات الدقيقة الموجودة فى المادة الغذائية إلا أنها طرق تستغرق الكثير من العمل والوقت اللازم فى إعداد البيئة وتحضين الأطباق وعد المستعمرات فضلاً عن الإنتظار لعدد من الأيام للنمو والحصول على النتائج. وبالإضافة لهذه المعوقات فإن هذه الطرق التقليدية بإستخدام الأطباق تتطلب فى بعض الأحيان تداول واستخدام مواد كيميائية شديدة الخطورة والتى تسبب خطورة بالغة للإنسان والبيئة عند تداولها والتخلص منها كما هو الحال عن تقدير البكتريا المسببة للأمراض. ولهذا فقد أجريت هذه الدراسة بهدف إستخدام طريقة التحليل الطيفى كوسيلة غير محطمة للعينة لإكتشاف وتقدير النمو الفطرى فى الخبز كواحدة من أكثر الطرق الواعدة فى هذا المجال. ولقد تم تحليل البيانات المستخرجة من شرائح الخبز بإستخدام طريقتى المحاور المتعامدة و أقل التربيعات الجزئية حيث أوضحت النتائج أن تخزين الخبز فى بيئة عالية الرطوبة لمدة أربعة أيام لم يكن النمو الفطري ملحوظاً ولكن مد فترة التخزين بعد هذه المدة سيؤدى إلى تغيراً جذرياً فى خصائص الخبز نتيجة لنمو الفطريات والتى تؤدى بدورها إلى تغيير صفاته وقابليته للأكل. ولهذا أمكن من خلال إستخدام التغير فى الخصائص الطيفية التمييز بين عينات الخبز المصابة بالفطر. ولقد أوضح تحليل أقل التربيعات الجزئية بإستخدام ٢٣٧ طول موجى وكذلك باستخدام أهم تسعة أطوال موجية على دقة تقدير النمو الفطرى فى شرائح الخبز بمعامل تقدير 0.97 و 0.94 فى عينات تجريبية وأخرى تأكيدية مما يعطى مؤشراً للتغيرات التى حدثت فى عينات الخبز نتيجة لنمو الفطريات بها. ونظراً لكون الطريقة المقترحة غير محطمة للعينات فهذا يجعلها ذات صدى علمى كبير لكونها قادرة على تحسين إسلوب إكتشاف العمليات المعقدة مثل تأثير النمو الفطرى فى الأغذية المختبرة. وعلى حسب معلوماتنا فإن هذه الدراسة تعتبر الأولى من نوعها لتطبيق تقنية التحليل الطيفى لإكتشاف النمو الميكروبى فى الخبز مما يفتح المجال للعديد من التطبيقات والتى تحتاج للكثير من البحث وذلك من خلال الإختبار على مدى أوسع من عينات الخبز المختلفة تحت ظروف تخزينية متنوعة وذلك بغرض التأكد من دقة وفاعلية الطريقة المقترحة ونماذج تحليل البيانات المستخرجة منها.

**قام بتحكيم البحث**

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| **أ.د / ممدوح محمد ربيع** | **كلية الزراعة – جامعة المنصورة** |
| **أ.د / رمضان أحمد عبد الغنى حبيبة** | **كلية الزراعة – جامعة قناة السويس** |