*Application of Support Vector Classification Algorithms for the Prediction of Quality Level of Frozen Shrimps (*Litopenaeus vannamei*) Suitable for Sensor-Based Time-Temperature Monitoring*

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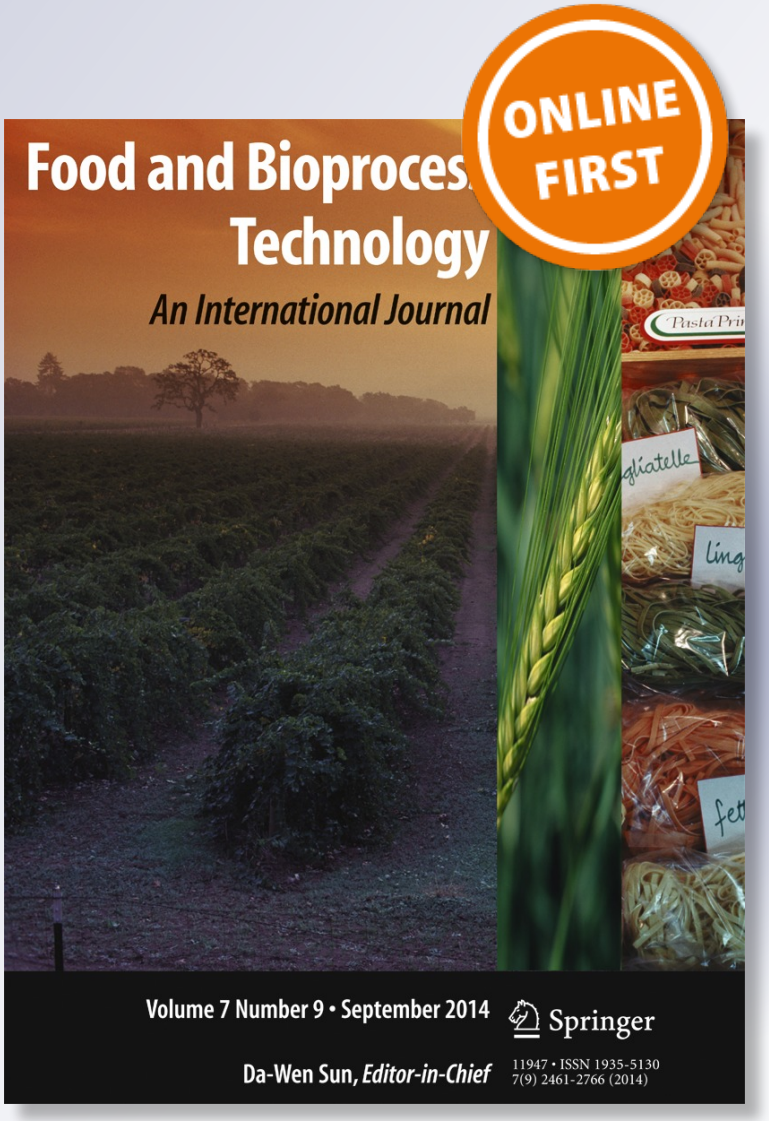
Food and Bioprocess Technology
An International Journal

ISSN 1935-5130

Food Bioprocess Technol
DOI 10.1007/s11947-014-1377-8



Springer

Food Bioprocess Technol
DOI 10.1007/s11947-014-1377-8

ORIGINAL PAPER

Application of Support Vector Classification Algorithms for the Prediction of Quality Level of Frozen Shrimps (*Litopenaeus vannamei*) Suitable for Sensor-Based Time-Temperature Monitoring

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Received: 14 January 2014 / Accepted: 16 July 2014

Abstract Classification of frozen shrimps (*Litopenaeus vannamei*) at the end-of-storage period in terms of physical, chemical, microbial, and sensory attributes is demonstrated in this paper. The quality parameters of frozen shrimps stored in isothermal and fluctuating temperature conditions were found significantly different ($p<0.05$). In order to classify products into binary classes of quality due to abusive and non-abusive handling (1, −1), useful features of the time-temperature curve were extracted to train the network using support vector classification (SVC). It was generally possible to classify shrimps based on end-of-storage quality by the available kernels; however, the radial basis function (RBF) was found to be the most suitable in terms of best fit and lower cost of calibration. The evaluation of the SVC kernels was done in terms of sensitivity and specificity values which were 0.962 and 0.992 in the case of RBF, respectively, whereas other kernels, polynomial and sigmoid, showed signs of over-fitting. The classification models were selected on the basis of root mean squared error (RMSE), mean absolute error (MAE), and other standard error measuring procedures. An independent cross validation procedure of the developed models was performed using a different set of data ($n=18$) against the model predicted values which lower root mean squared error (RMSE) terms (0.026–2.44) for all the quality indices, which confirmed the validity of the SVC technique.

Keywords Frozen shrimp · Quality classification · SVC · SVM · Quality management · Shelf-life · Cold chain

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Nomenclature

T	Temperature (°C)
FR	Freezing rate (°C min^{-1})
TR	Thawing rate (°C min^{-1})
WR	Warming rate (°C min^{-1})
RCR	Re-cooling rate (°C min^{-1})
t	Storage time (days)
AuC	Area under curve
SVM	Support vector machines
SVC	Support vector classification

Quality Parameters

L^* a^* b^*	CIE color parameters
H	Hardness (N)
Co	Cohesiveness (dimensionless)
Ch	Chewiness (N.s)
TVC	Total viable count (log of colony forming Units/g of sample)
TVB-N	Total volatile basic nitrogen (mg/100 g)
SI	Sensory index
ε	SVC tuning parameter
γ	Influence of a single training example
x	Vector
C	Cost of wrong predictions (penalty)
α	No. of samples × C
d	Distinct classes
Coef0	Parameter to define γ in sigmoid and polynomial kernels
SV	Number of support vectors used
TP	True positive predictions
FP	False positive predictions

Published online: 14 August 2014

TN True negative predictions
FN False negative predictions

Introduction

The frozen shrimp market is worth multibillion US$. About 80–90 % of the shrimp is exported from Asian countries to the US and European markets (Thai Frozen Food Association 2013). In order to supply high quality shrimps, farming and handling practices are under strict scrutiny more than ever before. Traditionally, the quality of frozen products is monitored by measuring environmental factors such as temperature and time spent in the cold chain. Efficient temperature monitoring across the supply chain and making use of the collected data have been a challenge for cold chain management. With the rise of modern embedded temperature sensors and data acquisition systems, it is now possible to implement an improved monitoring and analysis system. However, this requires robust and efficient models that can predict quality level from the time-temperature history during the supply chain stages (Ruiz-Garcia et al. 2008). There is a direct relationship with the loss of quality and fluctuation of storage temperature which has been mathematically modeled by a number of researchers (Giannakourou et al. 2006; Taoukis et al. 1991). Kennedy (1997) demonstrated that mild temperature abuse during storage had a significant effect on final quality of frozen products including seafood. In order to predict the level of quality at the end-of-supply chain, it is essential that the temperature fluctuation pattern is recorded to ascertain temperature abuse during transportation and storage. Using this information, classification models of frozen shrimps undergoing fluctuating and stable temperature regimes during sub-freezing storage and transportation in terms of quality indices such as CIE color parameters (L^*, a^*, b^*), textural profile analysis (TPA), total volatile basic nitrogen (TVB-N), total viable count (TVC), and sensory index (SI), are proposed in this paper.

The empirical and static nature of the most available quality prediction models limits their use. The models are often found to be unsuitable for online quality assessment schemes. Moreover, the mathematical models lack the ability of generalization, i.e., to accurately predict in varying environmental conditions, which is often the case with the large amount of data generated in a real world problem, such as time-temperature monitoring of frozen shrimp during distribution. In addition to mathematical and statistical tools to interpret quality classification, dynamic modeling of quality characteristics of frozen shrimps offers easy application and deployment of models. The dynamic modeling, also known as generic modeling, offers simple and robust models with high generalization ability. An artificial neural network (ANN) has been employed as an alternate to mathematical modeling, such as feed forward multilayer perceptron, back-propagation learning algorithm, etc., with high accuracy and generalization ability (Enitan and Adeyemo 2011). The commonly used ANNs, however, have been criticized for the "black box" nature of the models in which desired values can be predicted without having to comprehend the underlying physical processes (Paliwal and Kumar 2011). Moreover, other potential drawbacks of ANNs such as over-fitting, slow threshold convergence, and the possibility of falling in local optima, limit their practicality. Ahmad et al. (2013a, b) employed genetic algorithm (GA) to optimize ANN parameters such as learning rate, step size, and weight change momentum that are known to influence output response of the network. Storage period, freezing/thawing history, and geometric dimensions were used as input variables to predict output variables i.e., color ($L^*a^*b^*$) and texture profile analysis (TPA) parameters. Simple network architecture with one hidden layer (three to 17 nodes in each hidden layer) was obtained. Evaluation of the developed model was cross validated which yielded satisfactory results. The study concluded that a large set of data is required to obtain meaningful results. Moreover, the analysis was performed on continuous variables with the aim to predict time dependent variables. However, situations where categorical response is required, especially with limited amount of data and an overlapping decision boundary, ANN are known to yield inconsistent results (Ahmad 2013). ANNs are designed to reach a desired solution by solving a non-convex, unconstrained minimization problem during the network training. Owing to requiring large set of data for training has another implication that it might start to mimic the noise present in data as a part of pattern. Due to these reasons, it is rational to further explore the applicability of other available tools that are compatible with the online implementation arrangements.

A support vector machine (SVM) algorithm, originally proposed by Vapnik (1995), is based on statistical learning theory. With a strong mathematical basis, SVM is a new contender in data classification and time series prediction (Wu et al. 2009). SVM has excellent generalization ability and unlike ANN, it does not require a large set of data. In statistical learning theory, the basic concept of modeling is to select a model from hypothesis space which is in close proximity to the target space. Mismatch of target and hypothesis space may be associated with the approximation or estimation errors which add up as generalization error (Vapnik 1995). The approximation error measures fitness of the function as approximated by the learning system, whereas, the estimation error measures accuracy of the best function applied by the learning system using example of patterns (training data set). These errors are determined by the capacity, or lack thereof, of a pre-specified class of potential classifiers. With the SVM approach, the weights of the network are obtained by solving a quadratic programming problem with linear constraints.

Therefore, the optimization problem to be solved with support vector machines is set as a convex quadratic problem with a target to reach a global minimum, a distinct advantage over its contenders. Essentially, ANNs and SVM are closely related in the hierarchy of evolutionary algorithms; the use of a kernel function (described in next section) provides an unconventional training technique for polynomial, radial basis function and multilayer perceptron classifiers.

A number of researchers have reported the use of dynamic models for the prediction of physical properties and quality parameters of frozen shrimp (Goni et al. 2008; Mittal and Zhang 2000; Ahmad 2013). However, application of classification algorithms from a machine learning approach, as a possible solution to sensor-based supply chain monitoring of frozen products, has not been conducted.

Therefore, the objectives of this paper is to demonstrate the effect of temperature abuse during handling (storage and transportation) on the final quality of frozen shrimp and use the performance evaluation of support vector classification (SVC) algorithm to classify the final quality of frozen shrimp into two classes (1 and −1, representing high and low qualities, respectively) using time-temperature history.

Materials and Methods

Freshly harvested white shrimps (*Litopenaeus vannamei*) were collected and transferred alive to a laboratory while submerged in cold, aerated water bins. The samples were washed with fresh water and chlorine (3 ppm). In order for uniformity, oversized and smaller-sized shrimps were discarded.

Temperature Measurement

After degutting, de-shelling, and glazing, the samples were sealed in polythene bags and stored at −23±0.5 °C in chest freezers (LCDF 220, Evermed, Italy). In order to record freezer ambient temperature, the freezers were connected with a K-type thermocouple and a data logger. Cooling and warming rates (CR, WR, respectively) were determined according to the definition (Eq. 1) given by the IIR (1986):

$$\text{CR or WR} = \frac{T_2 - T_1}{t_2 - t_1} \tag{1}$$

where T_1 is the initial freezing temperature, T_2 is the final freezing point, and t_1, t_2 are the time intervals elapsed between the beginning and the end of freezing or thawing. A needle-like glass tube was inserted into the geometric mid-point of shrimp flesh. The thermocouple wire was securely attached inside the glass needle to ensure the temperature gradient is uniformly recorded. The purpose of glass tube was to serve as electric insulator to protect thermocouple from interference from any electric field that may cause noisy data recording. In this way, accidental fluctuation in temperature measurement was minimized. In addition, normalization procedure on temperature output was carried out to take into account the gradient, minimum and maximum temperature with respect to set point and ambient temperature according to the formula (Eq. 2):

$$\Delta T = \frac{T_i - T_s}{T_a - T_s} \tag{2}$$

where:

ΔT Normalized temperature difference, (°C)
T_i Temperature recorded at time "t", (°C)
T_s Temperature set point, (°C)
T_a Ambient temperature, (°C)

Even though the freezing temperature of shrimp is −2.2 °C, commercial processors usually freeze shrimp to −23.3 °C. This is because all free water in shrimps is frozen at this temperature (Rahman et al. 2003). Another practical reason to freeze to −23 °C is that after freezing, glazing process warms up the shrimps to −18 °C which is the average temperature of commercial freezers. As it is well known that about 25 % of water is unfrozen at −5 °C and even at −18 °C, 10 % water is still unfrozen that may contribute to oxidative chemical reactions (Elliot and Michener (1961), Huss (1995), Global Aquaculture Alliance (2008)). Therefore, −23 °C is a good choice if chest freezers are to be used. This point of temperature maintains quality as well as it is economical to operate. In addition, IIR (1986) recommended the practical storage life (PSL) of frozen shrimps as 2 and 5 months on −18 and −24 °C, respectively. The current study aimed at 3-month storage; therefore, a temperature of 23±0.5 °C was opted.

In order to mimic a supply chain scenario, firstly, samples were frozen to the desired freezing temperature (−23±0.5 °C) and then were allowed to spend 8 h at −10±0.5 °C at, randomly chosen, two different occasions during the entire storage period. Extreme abuse was not practiced as this is an unlikely scenario in a real supply chain. It took 8–10 h to warm samples from −23±0.5 °C to −10±0.5 °C. After spending 8 h at −10±0.5 °C, it took another 6 h to reach −23±0.5 °C where they spent the rest of the storage period. As it appears that temperature transitions used were quite slow, it is due to the fact that equipment used in supply chain (refrigerated transport, warehouses, etc.) is not meant for freezing, rather it is designed to maintain desired temperature. Therefore, in case an interruption in freezing storage occurs, the products are likely to take long time to reach to the desired temperature. The typical average weekly temperature is shown in Fig. 1. The storage period lasted for 90 days during which samples

were drawn after every 12 days for quality assessment, described in the next section.

Quality Assessment

Standard quality indicators routinely monitored in the frozen shrimp industry were selected, namely color (L^*, a^*, b^*), texture (hardness, cohesiveness, chewiness), total viable count (TVC), total volatile basic nitrogen (TVB-N), and sensory index (SI). All experimental observations were carried out in triplicate, and results are reported with standard deviations and appropriate error analysis as detailed in the following sections. Details of methodology of measuring these parameters are not included due to space limitation. An overview of each method, definition, and references are given in Table 1.

Preparation of Database

The entire temperature fluctuation pattern was used as input variables to predict the end-of-storage quality level of each parameter under investigation. In order to reduce the high number of data points of independent (input) variables, i.e., time and temperature, important features of the temperature curve (such as initial freezing rate and thawing rate of first fluctuation cycle, rate of re-cooling (°C.min^{-1}) of first and subsequent warming and final thawing), were identified and extracted. Freezing, re-cooling, warming, and thawing rates (FR, RCR, WR, and TR, respectively) were determined according to the definition given by the International Institute of Refrigeration (1986). In addition, a definite integral, in terms of area under the curve (AuC), of each curve, was obtained. Since the data points were in both negative and positive domains of the y-axis, therefore, the trapezoidal rule (Atkinson 1989) was applied in each segment of the curve and later, calculated areas under each segment were summed up. For training the network, these features along with point values of temperature at the onset of each segment of supply chain (T_1 to T_{12}, °C representing temperature at week 1 and week 12 of storage, respectively) were used as input variables against an output binary class variable which corresponds to the quality level. The range of minimum to maximum values of all input variables and desired outputs are given in Table 2. For all the quality parameters, a cutoff value in each quality parameter, corresponding to two quality classes (high and low), was determined (Table 5).

Support Vector Machines

SVM is a general term applied to both regression and classification problems. In this study, the term support vector classification (SVC) has been used to be specific. A schematic representation of SVC application for quality assessment is shown in Fig. 2. The variables in the input layer (T, FR, WR, RCR, t, and AuC) were mapped using a kernel function into a higher dimensional space separating into a binary output classes. The governing equation separating two different classes is as follows (Eq. 3):

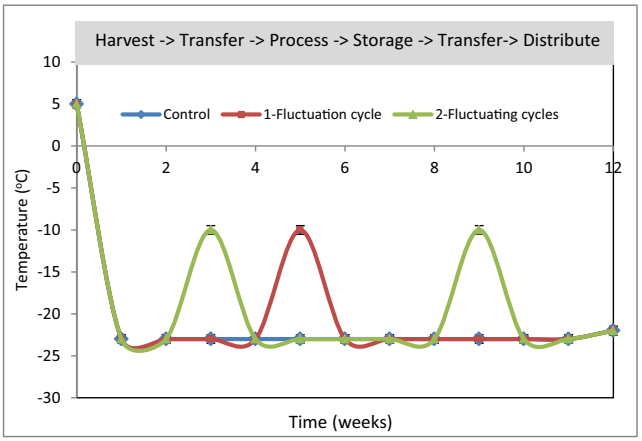

Fig. 1 Typical temperature fluctuation pattern of fresh shrimp used in this study

$$y(x) = w^T \varphi(x) = \sum_{j=1}^{K} \omega_j \varphi_j(x) + \omega_0 = 0 \quad (3)$$

where:$\varphi(x)=[\varphi 0(x), \varphi 1(x),\ldots,\varphi K(x)]^T$ with $\varphi(x)=1$ and w is the weight vector of the network, $w=[\omega_0, \omega_1,\ldots,\omega k]^T$.

The function φ (x) serves to plot n-dimensional input vector x into a K-dimensional feature space ($K>n$). The data vector x meeting the condition $y(x)>0$ falls in one category, whereas if $y(x)<0$, it will belong to the opposite category. If it is not possible that data is linearly categorized, a kernel function is used to plot the input data to a higher space to create two distinct classes. The kernel is defined as $K(x,x_i)=\varphi^T(x)\varphi(x_i)$. In this study, the commonly used kernels such as linear, radial basis function (RBF), polynomial kernels, and sigmoidal kernels were tested for their suitability on the same dataset. A definition of each kernel is given in Table 2. The learning vectors x_i is divided into two classes ($d_i=1$ or $d_i=-1$) by maximizing separation of margins, i.e., Q (∝) and is given by the following:

$Q(\propto)=\sum_{i=1}^{p} \propto_i -1/2 \sum_{i=1}^{p} . \sum_{j=1}^{p} \propto_i \propto_j d_i d_j \; K(x_i x_j)$ subject to constraints:

$\sum_{i=1}^{p} \alpha_i \alpha_j = 0$, $0 \le \alpha_i \le C$ and the resulting SVM takes the form of Eq. 4:

$$f(\mathbf{x}) = \mathbf{w}^T \Phi(\mathbf{x}_i) + b = \sum_{i=1}^{N} \alpha_i d_i K(\mathbf{x}_i, \mathbf{x}) + b \quad (4)$$

where C is a constant parameter set manually to regularize balance between training error and the model complexity, whereas p is the number of learning data pairs (x_i, d_i). The value of C is determined by trial and error. The value of C controls the compromise between training errors and overfitting by creating a soft margin that allows some

Table 1 Overview of quality assessment methods used in this study

Quality assessment	Description and unit	Instrument used	Reference
Color ($L^*a^*b^*$)	CIELab values (L value: lightness, a value: redness and greenness, b value: yellowness and blueness)	ColorFlex by Hunter Lab, USA	CIE (Commission International de l' Eclairage)
Total change in color (ΔE)	$\Delta E = \sqrt{(L_t - L_0)^2 + (a_t - a_0)^2 + (b_t - b_0)^2}$	ColorFlex by Hunter Lab, USA	Ahmed and Shivhare (2001)
Texture profile analysis (TPA)	Hardness (N) Cohesiveness (dimensionless) Chewiness (N.s)	TA-XT Stable Micro Systems, UK	Bourne (1978)
Total viable count (TVC)	log CFU/g	Microbiological assay	Maturin and Peeler (1998)
Total volatile basic nitrogen (TVB-N)	Lucke and Geidel's micro distillation Measured as mg of nitrogen/100 g of sample	Chemical assay	Pearson (1973)
Sensory index (SI)	Scoring on a scale of 1–3 (1 represents least preference and 3 for high preference)	Panel tasting	Huss (1995), Kreyenschmidt (2003)

misclassifications. If the value of C is increased, the cost of misclassifying points would also increase, leading to overfitting tendency and eventually a model with less generalization ability will be formed.

The algorithm performed a pattern search started from a specified data range in each direction. The search center was shifted to a new point if the model fits well. This process was repeated until no better fit found. At this stage, the search process is started over again with a reduced step size. The process continues by reducing the step size until it matches the specified tolerance limit.

Pre-treatment of Database

In order to apply classification algorithms, pre-processing of the dataset was carried out. The experimentally obtained 400 data rows (patterns) of input and output variables were used for training the algorithm. The task of model development was carried out in three phases: training, validation, and testing. Each data row or a pattern served as a training example. The data rows were randomized and divided into three groups: training dataset (200 examples), cross validation (c.v.) dataset (100 examples), and testing dataset (100 examples). In order to overrule accidental bias in the dataset, the network was developed by sixfold combinations of training, c.v., and testing datasets as depicted in Table 3.The range of input and output variables used for training algorithms is shown in Table 4.

Table 2 Definitions of kernels used in this study

Kernels	Kernel functions
Linear	$K(x,x_i)=(x^T.x_i+\gamma)$
Radial basis kernel (RBF)	$K(x,x_i)=\exp(-\gamma.\|x-x_i\|^2)$
Polynomial kernel with degree p	$K(x,x_i)=(x^Tx_i+\gamma)^p$
Sigmoid	$K(x,x_i)=\tanh(\beta_0 x^T x_i+\beta_1)$

As the kernel values are set according to the data points, which may lead to accidental bias due to imbalance, therefore, scaling of the dataset values of each attribute was set within the range of −1 to +1.

In addition, to reduce redundancy in the dataset, the values were normalized by finding the cumulative mean of all preceding values. For instance, the first normalized value was the average of first two values, and the second normalized value represented the average of the first three values, and so on. The preceding uniformly spaced values were then divided by the first value of the column which yielded, 1 and preceding values in the column were always <1.

Statistical Analysis and Evaluation of Classification Models

Analysis of variance (ANOVA) and least significant difference (LSD) were employed to determine statistical significance of experimental values using SPSS ver. 12 software package.

The suitability of a classification algorithm was determined in terms of a penalty cost (C), i.e., the cost of wrong predictions, %error, and %correct classification. Overall, support vector classifier performance was measured in terms of sensitivity (Eq. 5) and specificity (Eq. 6):

$$\text{Sensitivity} = \frac{\text{True positive predictions}}{\text{True positive predictions} + \text{False negative predictions}} \quad (5)$$

$$\text{Specificity} = \frac{\text{True negative predictions}}{\text{True negative predictions} + \text{False positive predictions}} \quad (6)$$

Sensitivity is defined as the part fraction of positive examples that were correctly retrieved. On the other hand, specificity is given as the part fraction of negative examples that were correctly retrieved. Sensitivity describes the effectiveness of the classifier at reaching a region of interest in the dataset. The specificity characterizes the ability of the input variable to reject non-qualifying examples. In this way, an SVC kernel

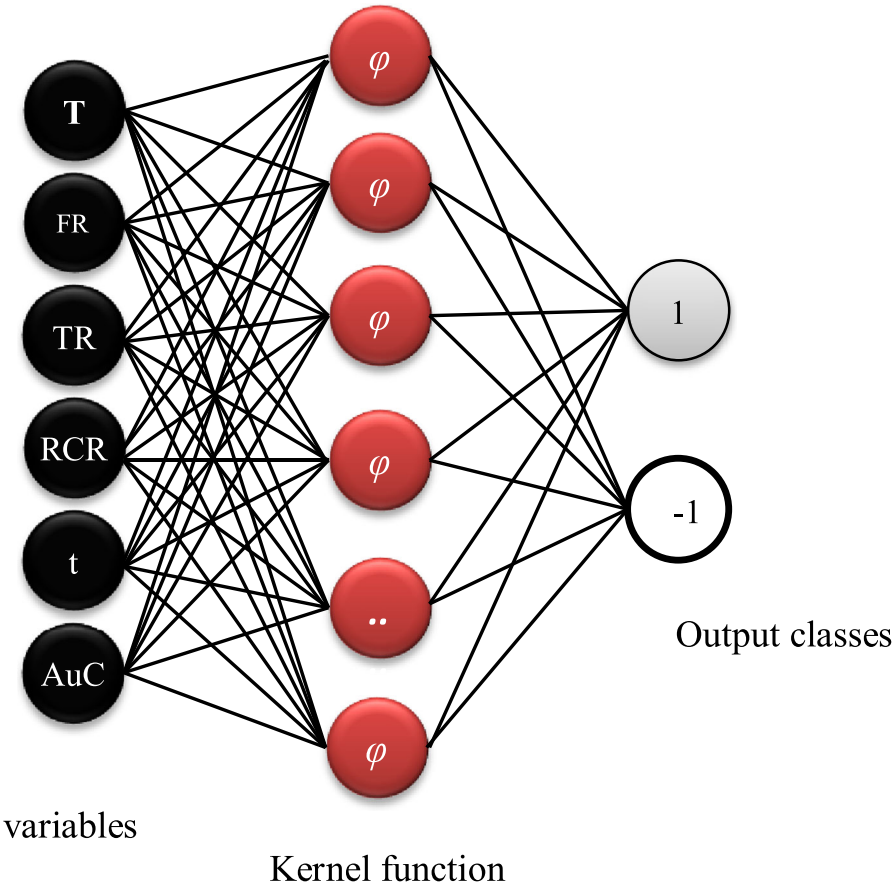

Fig. 2 Schematic representation of quality level classification using support vector algorithm based on time-temperature history of frozen shrimp during distribution

that produces high sensitivity values and at the same time maintains a good range of specificity values in the solution of a problem ensures model stability.

Performance of network was evaluated using the average error and the mean squared error (MSE) in training, validation, and test data sets. Definition of both indicators is given below (Eqs. 7 and 8):

$$\mathrm{MSE} = \frac{1}{n}\sum_{i=1}^{n}\left(\widehat{y}_i - y_i\right)^2 \tag{7}$$

where $\widehat{y}_i, y_i$ are computed and observed values of n cases and:

$$\text{Average Error} = \frac{\text{Sum Squared Error}}{n} \tag{8}$$

The average error depicts the average sum of squares of residuals in the model (training dataset in this case) based on total sample size. The model predicted values were compared against observed values in terms of root mean squared error (RMSE) (Eq. 9):

$$\mathrm{RMSE} = \frac{1}{N}\sqrt{\left(\widehat{y}_i - y_i\right)^2} \tag{9}$$

For independent validation of developed model, NMSE (normalized mean squared error), MSE (mean squared error), MAE (mean absolute error), and MAPE (mean absolute percentage error) were used. An evaluation version of DTREG (2013), predictive modeling software was used to perform SVC analysis.

Table 3 Arrangement of dataset for training the network (number of rows)

	Arrangement sets of data rows					
	1	2	3	4	5	6
Training dataset	1–200	101–300	201–400	1–200	101–300	201–400
C.V. dataset	201–300	301–400	101–200	301–400	1–100	1–200
Testing dataset	301–400	1–100	1–200	201–300	301–400	101–200
Average training error	0.063	0.049	0.045	0.031	0.06	0.053

Table 4 Range of input and output variables used for training algorithms (n=400)

	Minimum (SD)[a]	Maximum (SD)[a]	Mean	Over all SD[b]
Input variables				
Temperature (°C)	−23±0.5	−10±0.5	−16.5	0.5
Freezing rate (°C.min^{-1}), FR	0.2±0.02	0.6±0.07	0.4	0.07
Thawing rate (°C.min^{-1}), TR	3.3±0.3	5.8±0.5	4.55	2.4
Re-cooling rate (°C.min^{-1}), RCR	0.22±0.02	0.99±0.089	0.605	0.3
Storage time (days), t	12	96	54	NA
Area under curve (m^2)	0.0448±0.005	0.186±0.02	0.1154	0.0368
Output variables				
L*	24.0±1.1	33.0±0.5	28.5	3.2
a*	−1.67±0.15	1.12±0.15	−0.275	0.31
b*	1.63±0.2	6.45±0.26	4.04	7
Hardness (N)	70±14	130.8±13.1	100.4	15.4
Cohesiveness (dimensionless)	1.45±0.29	5.69±0.57	3.57	0.45
Chewiness (N.s)	41.3±8.2	102.6±10	71.95	1.45
TVC (log CFU/g)	4.32±0.22	6.46±0.56	5.39	0.74
TVB-N (mg/100 g)	7.2±0.07	26.3±0.4	16.75	3.43
Sensory index	3±0.25	2±0.25	2.5	0.44

[a] SD - standard deviation of measurement of replications

[b] SD - over all standard deviation of database (n=400)

Results and Discussion

The first part of this section establishes that the dataset contains two distinct classes by classical statistical methods. In the second part, application of the support vector classification algorithms is demonstrated.

Effect of Temperature Fluctuation Cycles

A temperature fluctuation in sub-freezing zone from −23±0.5 to −10±0.5 °C is considered mild temperature abuse (Kennedy 1997). However, this mild temperature abuse cycles showed significant changes in all the quality parameters under investigation (Table 6). The spoilage pattern for each quality parameter was the same for all samples. Samples in temperature-controlled regime showed the lowest change in quality level, samples undergoing two temperature fluctuation cycles showed the highest variation, whereas samples in one fluctuation cycle were intermediate. All results were significant at $p<0.05$.

Color The color parameters ($L^*a^*b^*$) were significantly different in all three temperature regimes. The difference started to be noticeable after 2 weeks of storage, except for a* value, which is less related with shrimp color. The lightness (L*) decreased from 30.4±1.3 to 24.3±0.22 in 2 cycles of fluctuation, which was the highest change, as compared to the change recorded in samples undergoing 1 cycle of fluctuation. Similarly, the b* value increased steadily in both cases. Homogeneity tests (Fisher's least significant Difference (LSD)) along with ANOVA analysis showed that all three groups were significantly different at p=0.05, with high F

Table 5 Cutoff values of quality parameters of frozen shrimp corresponding to binary classes

Quality parameter	High quality (1)	Low quality (−1)	Reference
Color			
L*	≥51	≤50	NA
a*	≤5.9	≥6.0	
b*	≥9.1	≤9.0	
Texture			
Hardness (N)	≥51	≤50	NA
Cohesiveness (dimensionless)	≥1.0	≤0.9	
Chewiness (N.s)	≥83	≤82	
TVC (log CFU/g)	≤7.4	≥7.5	Maturin and Peeler (1998)
TVB-N (mg/100 g)	≤25	≥26	Einarsson (2001)
Sensory index (1–3)	≥1.9	≥1.8	Huss (1995)

Table 6 Values at the end-of-storage period for quality parameters of frozen shrimps subjected to two temperature scenarios for a period of 3 months

Parameters	Control	Fluctuating (1 cycle)	Fluctuating (2 cycle)	*F*	LSD (SE)
Color					
*L**	30.35±1.3[a]	27.97±0.25[b]	24.3±0.22[c]	58.952	6.85 (0.67)
*a**	0.13±0.02[a]	0.62±0.03[b]	0.87±0.035[c]	425.530	28.9 (0.02)
*b**	3.05±0.26[a]	4.28±0.4[b]	6.25±0.05[c]	100.588	17.88 (0.02)
ΔE	5.78±0.34[a]	7.24±0.02[b]	8.75±0.65[c]	204.277	6.47 (0.23)
Texture					
Hardness (N)	112.33±2.5[a]	83±3[b]	70±2[c]	218.914	14.15 (2.07)
Cohesiveness (dimensionless)	3.28±0.33[a]	1.87±0.11[b]	1.45±0.26[c]	42.353	6.78 (0.21)
Chewiness (N.s)	85.31±11[a]	42.36±3.2[b]	41.34±2.5[c]	4.807	2.65 (16.19)
Microbial quality log (CFU/g)	4.63±0.4[a]	5.6±0.45[b]	5.59±0.63[b]	6.834	3.28 (0.31)
TVB-N (mg/100 g)	17.6±0.8[a]	20.2±2 [b]	25±0.5 [c]	25.32	2.4 (0.9)
Sensory index	2.88±0.6[a]	2.4±0.13 [b]	1.9±0.55[c]	80.08	6.99 (0.07)

All data were subjected to analysis of variance (ANOVA), post hoc multiple comparison test in triplicate at 95 % confidence level ($\alpha<0.05$)

Superscript letters represent that the means were significantly different

Control regime: samples were kept at a constant temperature of −23±1 °C

Fluctuation regime: samples were subjected to −23 °C, − 10 °C, −23 °C (1 cycle) and −23 °C, − 10 °C, −23 °C, −10 °C, −23 °C (2 cycles)

LSD (*SE*) least significant difference (standard error)

value, low standard error, and significant LSD values (Table 5). This confirmed that both the length of storage and fluctuation in sub-freezing temperature regimes affect the change in color of frozen shrimp. This deterioration behavior of products took place due to stress damage and fat oxidation, induced by temperature fluctuation (Ahmad 2013).

The human perception of change in color is best represented by ΔE, which is the sum of $L^*a^*b^*$ values, in terms of their corresponding difference between day 1 and the final day of storage. The ΔE was recorded as 5.78±0.34, 7.24±0.02, and 8.75±0.65 in the control, for one and two fluctuation cycles, respectively. These ΔE values were significantly different (at $p=0.05$) with high F significance (Table 6). This change in color indicated loss in redness and overall color fading appearance of samples. Color loss is one of the adverse effects of frozen storage found in tuna (Chow et al. 2004), salmon (Sigurgisladottir et al. 1999), and shrimps (Chandrasekaran 1994).

Texture Temperature fluctuation cycles induced fat oxidation, protein denaturation due to breaking of ice crystals, and forming large ice crystals during subsequent re-freezing resulting in textural changes. The final hardness value at the end-of-storage period was 112.33±2.5, 83±3, and 70±2 N in the control, for one and two fluctuation cycles. Similarly, cohesiveness and chewiness decreased in the three temperature scenarios. As can be seen in Table 5, all three textural parameters in three regimes were significantly different ($p<0.05$). This is in line with the previous finding of Tsironi et al. (2009) in which an adverse effect of storage temperature on shrimps was reported; however, temperature fluctuation was not taken into account.

TVC The total viable counts (TVC) in all three temperature scenarios were in the range of 4±0.4–6±0.6 log[CFU/g]. Although, control samples had a significantly lower microbial population than samples in fluctuating regimes, the number of fluctuating cycles had no effect on population growth. The rise in TVC could be attributed to the batch effect. Microbial load in terms of TVC of thawed control samples was 4.63 log CFU/g which is lower than the ones reported by Tsironi et al. (2009) and Jeyasekaran et al. (2006) but within the same range of some other studies such as Hatha et al. (1998), indicating hygienic handling conditions were practiced. The load of thawed samples of both fluctuating cycles were higher (5.6 log CFU/g) than that of control samples. Hossain et al. (2010) have reported a total microbial count of 10^4 after freezing raw black tiger prawns at −38 °C in block form of freezing; however, the total microbial load reduced to 10^3 when individual quick frozen technique (−40 °C) was used. Possible explanation of survival of microorganisms at low freezing temperature is given by Tsironi et al. (2009). Some bacterial species such as *Psychrobacter* are psychrotolerant found in marine environments and has been reportedly responsible for seafood spoilage, particularly, shrimp that despite optimally frozen in commercial handling can also be seriously degraded by months of frozen storage under fluctuating temperature (Global Aquaculture Alliance 2008). In addition, as the number of total microbial load varied between 4 and 6 log CFU/g, it could be due to re-

contamination during final thawing before quality analysis and might also be due to the packaging material. In accordance with the results reported by Tsironi et al. (2009), the samples were not appeared spoiled despite the slow growth observed at the end-of-storage period.

TVB-N The 2-cycle fluctuating regimes gave the highest change in TVB-N levels (25±0.5 mg/100 mg), followed by the 1-cycle fluctuation, and finally the control samples had the lowest change. It was obvious that during the first 2 weeks of storage, there was no change in TVB-N levels. Then, a linear increase in TVB-N levels was observed in all storage scenarios until 8 weeks of storage. TVB-N levels were found to increase at a higher pace in samples exposed to 2-fluctuations, corresponding to the second cycle. Nevertheless, the total change in TVB-N levels was within the acceptable range, i.e., 35 mg/100 g for frozen products.

Sensory Index Panelists were asked to rate coded samples on a scale of 1–3, where 1 represented the least preferred quality and 3 for the most desired quality. The quality parameters included in the tests were color, texture, and odor. From week 2 onwards, there was a clear difference in sensory score. Samples in all three temperature regimes were scored differently. All the 20 panelists who were asked to rank the samples, preferred samples stored at a stable temperature. However, there was some variation in samples of fluctuating regimes. Nevertheless, the rank sum showed temperature fluctuation is detectable by humans on a statistically significant scale. The spoilage trend of all abovementioned quality indices is shown in Fig. 3.

In conclusion, the temperature fluctuation cycles produced stress damage even though the abuse was mild, i.e., samples were never allowed warmer than −10 °C. The stress damage is attributed to fat oxidation, resulting in changes in color, texture, and sensory index. The effect of three temperature scenarios was more apparent for the quality parameters such as color and texture. A consistent pattern in changes of quality parameters in all three regimes was found, i.e., lowest changes for stable storage temperature, medium for 1-cycle fluctuation, and highest for 2-cycle fluctuation. These results were in accordance with Kennedy (1997) and Gormley et al. (2002).

Application of SVC Algorithm

In order to differentiate between products undergoing abusive and non-abusive temperature regimes, evaluation of various SVC kernel functions was determined. Comparison of kernel functions used to develop SVC models is shown in Table 6. According to the results, all kernel functions were able to correctly classify quality attributes in response to abusive and non-abusive handling. However, selection was made on the basis of three criteria, i.e., sensitivity, specificity, and whether or not algorithms showed signs of over-fitting.

Linear Kernel Optimum C was searched between 0.1 and 5,000. It was found that as the number of C increased, over-fitting occurred. Therefore, for linear kernel, C was set at 50.

Polynomial Kernel Coefficient 0 was searched between 0 and 100 and for 1–3° of polynomial. The algorithms performed best at degree 1 and C=100, after which over-fitting occurs.

Sigmoid A higher C than other kernels and abundant use of support vectors disqualified this kernel. Moreover, TP and TN were 100 % which is a clear sign of over-fitting of the model.

RBF Kernel The value of gamma was searched between 0.001 and 50, and the optimum value was found to be 0.12 and C=0.2. Again, with a further increase in value of C or a decrease in gamma, over-fitting occurred. There was a visible increase in performance of RBF kernel when gamma reduces or cost increases, with the mentioned values as the threshold.

Optimized parameters of all the kernels are given in Table 7. Although, the linear kernel yielded acceptable sensitivity and specificity values (0.897 and 0.94, respectively), a total of 84 support vectors were utilized by the model to train on patterns existed in the dataset. In the case of both polynomial and sigmoid kernels, there was a tendency of over-fitting owing to utilizing a high number of support vectors in the input space. The polynomial kernel does not work well for any degree greater than 1, after which it ended up using high number of the elements of the training data as support vectors and over-fits greatly. The sensitivity and specificity values were 1, which could be regarded as a sign of non-responsive model. The RBF kernel, on the other hand, utilized only 53 support vectors and the corresponding sensitivity and specificity values were in good balance. The RBF kernel appeared to be the most suitable kernel function on account of short training time, wider search space, right number of support vectors, and its generalization ability. This finding was in accordance with the recommendation of Hsu and Lin (2010) with respect to the use of RBF. The radial basis function (RBF) was preferred among other choices of kernel types due to its confined and uniform output responses throughout the entire data range of the dependent axis. The classification results of each quality attribute are shown in Table 7. These results confirmed that linear, polynomial, and sigmoid kernels were outclassed by the RBF kernel; therefore, the SVC model was built on RBF kernel with optimized parameters as ε=0.001, C=0.1, γ=0.1225.

For color parameters, sensitivity values of >90 % in training with specificity values in the same range in the training dataset showed promising results. The results were similar in the validation and test dataset, suggesting high reproducibility of the RBF model. In both training and test dataset, the b^* value tend to use a high number of support vectors as

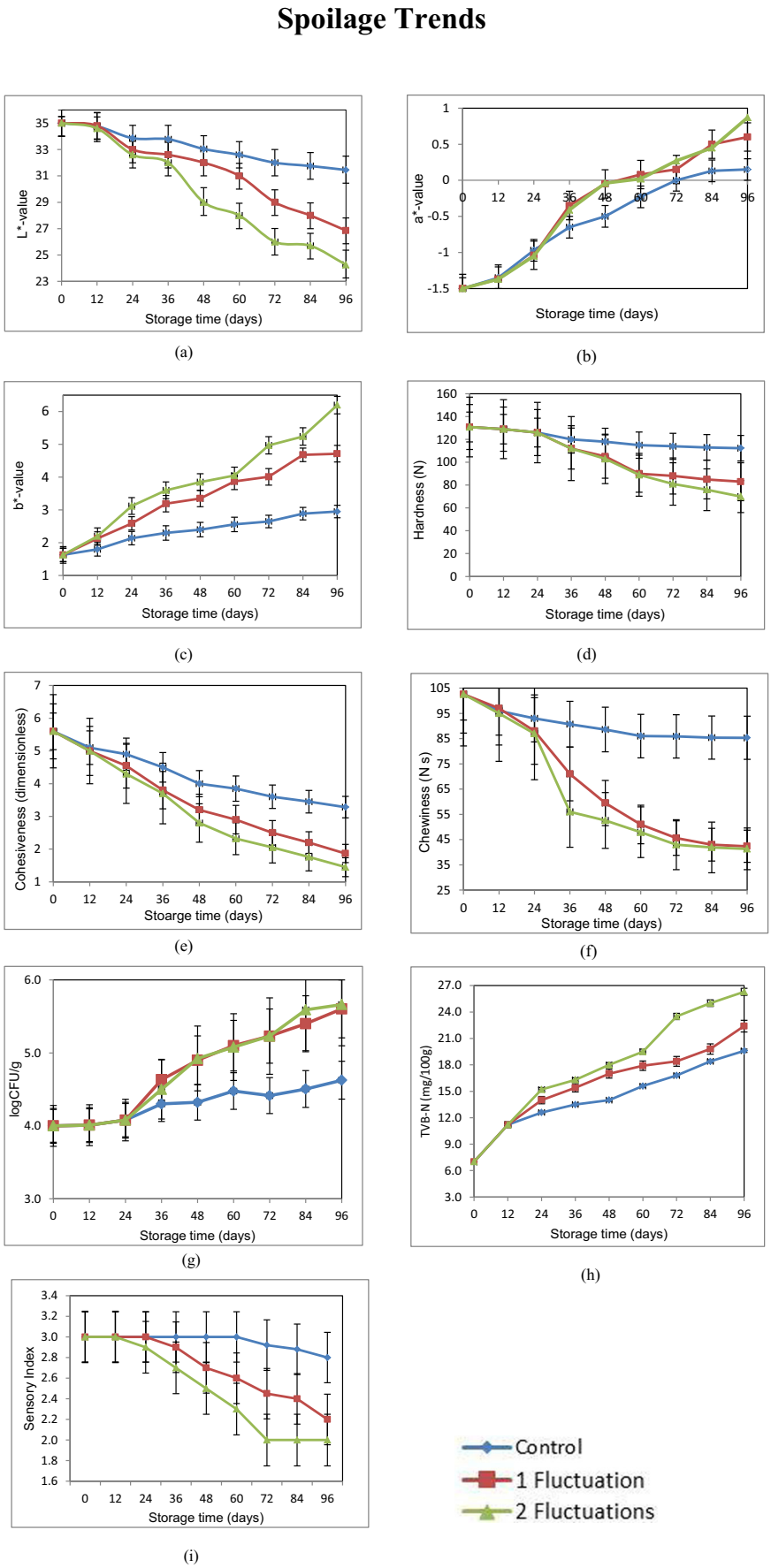

Fig. 3 Spoilage trends of **a** L^*, **b** a^*, **c** b^*, **d** hardness, **e** cohesiveness, **f** chewiness, **g** TVC, **h** TVB-N, and **i** sensory index

compared to L^* and a^*, which can be attributed to the existence of non-linearity in the data. In the test dataset, specificity values for a^* and b^* were found to be 66 and 73 %, respectively, which were lower than the values found in the training dataset. However, the corresponding values of sensitivity, %TP and %TN, provided a balance for selecting the model.

A similar trend was observed for textural properties, except cohesiveness, which tended to utilize a higher number of support vectors in the training dataset. TVB-N results were according to the industrial acceptance criterion and in accordance with the other studies (Tsironi et al. 2009; Salvi 2005; Zeng et al. 2005). In terms of predictive studies, Salvi (2005) reported a low R^2 when ANN (feed forward) was used to predict TVB-N in frozen shrimp. Zeng et al. (2005) has reported a good correlation between TVB-N and sensory evaluation; however, predictive analysis has not been conducted. TVC and SI, on the other hand, were found to utilize a higher number of SVs in the training dataset; however, the possibility of over-fitting was overruled as the model reached an optimum solution with a lesser number of SVs in validation and test datasets (Table 8). In addition, both the TVC and SI were relatively linear in nature and hence less complex than textural data. It was possible to classify shrimps on the basis of TVC, obtained in isothermal and non-isothermal conditions using Euclidean distance approach with 64 % accuracy (Salvi 2005), however, the study suffered with less number of data points. According to Bahamonde et al. (2007), SVC was the preferred method of classification in panel tasting procedures due to reasons such as large sample size and inability of panelists to taste all samples in a short time, which lead to missing datasets. In most cases, subjective assessment leads to relative scoring. In order to avoid relative scoring, SVC can effectively differentiate the decision boundaries (Shawe-Taylor and Cristianini 2004).

Working Equation of SVC Model

By combining Eq. 1 and RBF kernel expression, the functional form (Eq. 10) of model is represented as follows:

$$\mathrm{SVC}(x) = \sin\left[\alpha_1 e^{-\gamma||x_1-x||^2)} + \alpha_2 e^{-\gamma||x_2-x||^2)} + \ldots + \alpha_n e^{-\gamma||x_n-x||^2)} + b\right] \tag{10}$$

where α and γ are model parameters, shown in Table 8, and x_1 to x_n represent support vectors, i.e., subset of training example. The total number of support vectors used for training each quality index is given in Table 7. However, all support vector values are not shown due to space limitation. With this information, a computer program can be coded to separate data into a higher dimensional space through a hyper plane.

Model Evaluation

Using an unseen set of data (n=18), the RBF model (Eq. 10) with the optimized equation constant parameters (ε=0.001, C=0.1, γ=0.1225), new quality parameter values were generated and compared against the randomly selected experimentally obtained values. A comparison of predicted and experimentally observed values was made in terms of RMSE which was 0.159, 0.49, 1.22, 0.026, 0.159, 0.08, 0.462, 2.44, and 0.23 for L^*, a^*, b^*, hardness, cohesiveness, chewiness, TVC, TVB-N, and SI, respectively. Among all, the

Table 7 Best performance results of kernel application obtained during training of all quality attributes (n=300)

Kernel	SV	TP (%)	FN (%)	TN (%)	FP (%)	Sensitivity	Specificity
Linear							
ε=0.001 C=0.1	84	89.7 %	10.26 %	94 %	6.10 %	0.897	0.939
Polynomial							
ε=0.001 C=0.1 γ=0.001 Polynomial degree=1–3	118	1	0	1	0	1	1
Sigmoid							
ε=0.001 C=0.332 γ=0.0368	224	1	0	1	0	1	1
RBF							
ε=0.001 C=0.2 γ=0.1225	53	96.2 %	3.8 %	99 %	1 %	0.962	0.992

SV number of support vectors used by the model, *Sensitivity* fraction of positive examples that are correctly retrieved, *Specificity* fraction of negative examples that are correctly retrieved

Table 8 Classification results of quality attributes of frozen shrimps obtained using RBF kernel function (n=200, 100, and 100 for training, validation, and training datasets, respectively)

Quality attribute	SV	TP (%)	FN (%)	TN (%)	FP (%)	Sensitivity	Specificity
Training(n=300)							
L^*	60	0.94	0.06	0.93	0.07	0.941	0.931
a^*	78	0.97	0.03	0.90	0.10	0.969	0.896
b^*	120	0.92	0.08	0.95	0.05	0.925	0.953
H (N)	40	0.89	0.11	0.98	0.02	0.891	0.983
Ch (Ns)	84	0.85	0.15	0.95	0.05	0.851	0.946
Co	112	0.92	0.08	0.92	0.08	0.923	0.919
TVB-N (mg/100 g)	72	0.98	0.02	0.99	0.01	0.978	0.986
TVC (log CFU/g)	102	0.98	0.02	0.96	0.90	0.983	0.516
SI	120	0.98	0.02	0.95	0.03	0.981	0.966
Validation (n=100)							
L^*	30	0.82	0.18	0.95	0.05	0.816	0.950
a^*	32	0.92	0.08	0.94	0.06	0.923	0.943
b^*	45	0.91	0.14	0.78	0.22	0.864	0.783
H (N)	35	0.97	0.10	0.93	0.07	0.906	0.933
Ch (Ns)	59	0.94	0.06	0.89	0.11	0.944	0.893
Co	39	0.97	0.03	0.85	0.15	0.970	0.848
TVB-N (mg/100 g)	49	0.99	0.01	0.90	0.10	0.988	0.900
TVC (log CFU/g)	51	0.90	0.10	0.75	0.25	0.897	0.750
SI	40	0.93	0.07	0.93	0.07	0.929	0.933
Test (n=100)							
L^*	29	0.93	0.07	0.80	0.20	0.933	0.800
a^*	35	0.95	0.05	0.67	0.33	0.954	0.666
b^*	49	0.99	0.01	0.73	0.27	0.988	0.733
H (N)	28	0.91	0.10	0.76	0.24	0.831	0.868
Ch (Ns)	33	0.88	0.12	0.76	0.24	0.831	0.868
Co	31	0.89	0.11	0.74	0.26	0.831	0.868
TVB-N (mg/100 g)	27	0.94	0.06	0.73	0.27	0.830	0.869
TVC (log CFU/g)	20	0.99	0.01	0.60	0.40	0.851	0.891
SI	17	0.95	0.05	0.92	0.08	0.852	0.890

RBF model parameters: ε=0.001, C=0.1, γ=0.1225

Table 9 SVM model evaluation (cross validation)

	L^*	a^*	b^*	H	Co	Ch	TVC	TVB-N	SI
CV	0.002	0.074	0.125	0.069	0.832	0.196	0.085	0.13	0.09
NMSE	0.0004	0.012	0.03	0.007	0.516	0.074	0.095	0.11	0.27
Maximum error	0.46	0.97	4.96	0.09	0.61	0.22	1.032	7.49	0.62
RMSE	0.159	0.49	1.22	0.026	0.159	0.08	0.462	2.44	0.23
MSE	0.025	0.24	1.5	0.0006	0.025	0.006	0.213	5.95	0.05
MAE	0.107	0.37	0.57	0.012	0.068	0.057	0.374	1.63	0.16
MAPE	0.203	11.71	5.85	6.457	25.45	16.19	7.135	8.58	7.03

Average results of threefold cross validation

CV coefficient of variation, *NMSE* normalized mean square error, *RMSE* root mean squared error, *MSE* mean squared error, *MAE* mean absolute error, *MAPE* mean absolute percentage error

TVB-N showed the highest error whereas the rest of the values were in acceptable range. Other error measuring terms are listed in Table 9, which further confirmed the validity of the developed model. Keeping in view, the classifier performance and cross validation results, it was concluded that the RBF classification model can satisfactorily be used for industrial purposes with high reliability.

Further to the authors' previous study (Ahmad et al. 2013a, 2013b), in which ANN (multilayer perceptron optimized with GAs) was used as an attempt to predict quality parameters of frozen shrimp, the current study offers an alternate to ANN. While it is not the scope of the study to argue algorithm superiority over each other, one of the distinct features of SVM makes it worthy for the investigation in comparison to ANN, i.e., non-parametric nature of an SVM model which consists of a set of support vectors with a weight value for each, selected from the training set. As long as an SVM model do not utilize a large number of support vectors during the training step, does not show sign of over-fitting during evaluation, and the classification performance measuring indicators (%FP, %TN, sensitivity and specificity) are in good balance, the model would make a good choice for online (sensor-based) implementation.

Conclusions

In general, this study demonstrates binary classification of frozen shrimp based on common quality attributes. Specifically, the study showed that mild temperature abuse at sub-freezing temperature is detectable, and products can be divided into two categories based on the end of supply-chain quality level obtained through time-temperature history data. Shrimps are particularly sensitive to temperature variations due to heterogeneous structure, denaturation of protein, sublimation, and re-crystallization of ice. Although, significant changes were noticed in TVB-N, TVC, and sensory index, those were within industrial acceptable limits. Mild temperature abuses may not be apparent at the end of supply chain, but the onset of spoilage reaction reduces the quality at a faster pace during retail distribution. Therefore, stable storage temperature is necessary for maintaining high quality throughout transportation and retail. The results have established that sub-freezing fluctuations have an adverse effect on quality, which lead to quicker spoilage of shrimps. By subjecting shrimps into different temperature fluctuation scenarios and resulting quality level could successfully be "learned" by a support vector classification (SVC) algorithms as a binary class output (low quality and high quality). Among the commonly available kernels, the RBF kernel models showed a higher generalization tendency. Moreover, the SVC-RBF was preferred due to properties such as lower calibration cost (time to train and test) and higher precision in prediction with less number of support vectors utilization during training of the algorithm. SVC is potentially a suitable method for the food industry, for subjective analysis (such as panel tasting), where difficulty is encountered due to overlapping decision boundaries.

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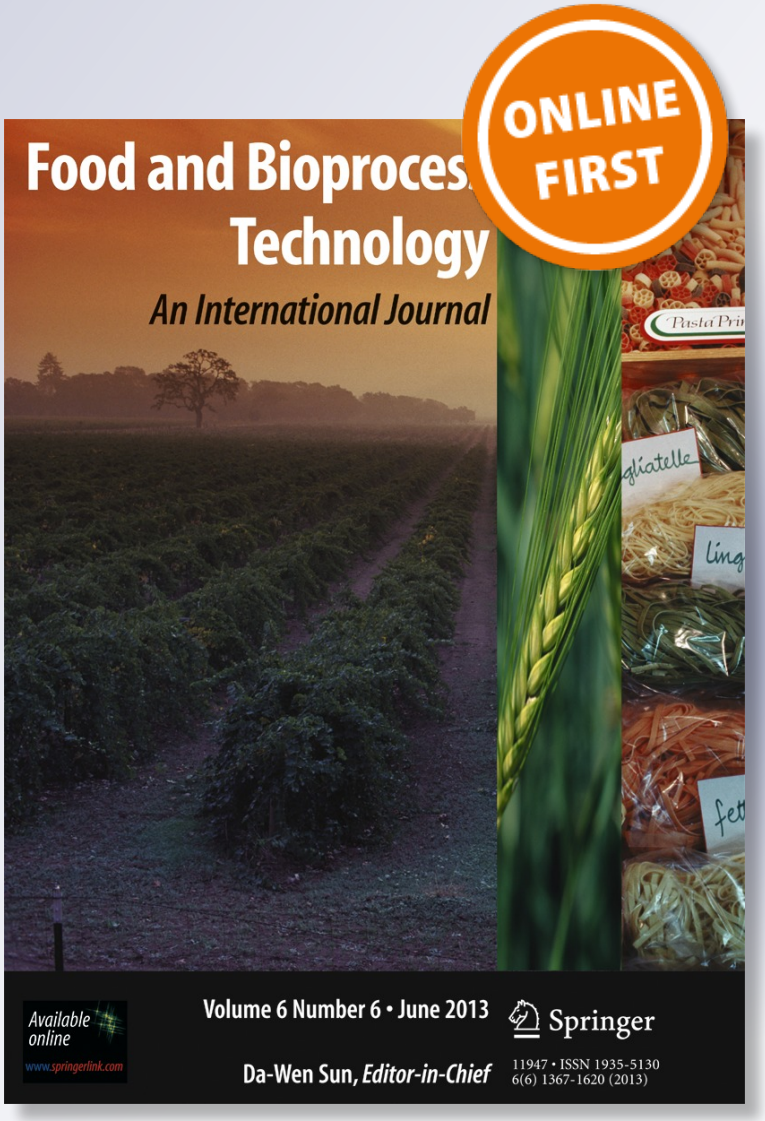
Food and Bioprocess Technology
An International Journal

ISSN 1935-5130

Food Bioprocess Technol
DOI 10.1007/s11947-013-1135-3



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DOI 10.1007/s11947-013-1135-3

ORIGINAL PAPER

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Received: 6 March 2013 / Accepted: 16 May 2013

Abstract Application of genetic algorithm to optimize an artificial neural network (ANN) model for predicting end-of-storage quality parameters of frozen shrimp (*Litopenaeus vannamei*), which influence consumer purchase decisions, is demonstrated in this paper. Freezing rate (FR), thawing rate (TR), storage time, width, thickness, and length of frozen shrimp were measured and chosen as input variables to train the ANN against Commission International de l' Eclairage Color *L*a*b** values, and textural properties (hardness, cohesiveness, and chewiness) as dependent variables. Experimentally obtained randomized data points (500) were used to develop the network, of which 20 % were used for testing the network, as an unseen environment. The developed genetic algorithm–artificial neural network (GANN) which included one hidden layer with 3–17 neurons successfully predicted color and textural values with correlation coefficient, R^2, of >0.9 and root mean square error (RMSE) of <1.6. The redness (*a**) and cohesiveness took the longest training time and highest number of generations, as compared to the other parameters. Percent relative importance of input variables to output variables indicated that TR, FR, and storage time were the most important variables for the prediction of color and texture parameters. The results are compared with multiple linear regression (MLR) and ANN trained with backpropagation (BP) algorithm. The results indicate that the GANN model shows much better prediction, as compared to MLR and BP with smallest RMSE and highest R^2.

Keywords Frozen shrimp · Freezing rate · Thawing rate · Storage temperature · GANN · Genetic algorithm · Artificial neural networks

Nomenclature and abbreviations

ANN	Artificial Neural Network
FR	Freezing rate (°C min^{-1})
TR	Thawing rate (°C min^{-1})
T_1	Initial product temperature (°C)
T_2	Final product temperature (°C)
t_1	Starting time for storage (min)
t_2	Ending time for storage (min)
*L*a*b**	CIE color parameters
TH	Thickness (m)
W	Width (m)
L	Length (m)
H	Hardness
Co	Cohesiveness
Ch	Chewiness
GA	Genetic Algorithm
MSE	Mean squared error
RMSE	Root mean squared error
x	Input neurons
y	Output neurons
b_{ij}	Bias
w	Weights

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Introduction

The frozen shrimp market is worth US$ 2.5 billion, and the demand for it continues to grow at a rate of 20 % annually. Consumers in importing countries are increasingly concerned with the quality of frozen shrimp (TFFA 2011).

Published online: 04 June 2013

This has necessitated the implementation of schemes for monitoring quality-affecting factors such as temperature, storage time, packaging, and handling. Temperature, being the single most important factor responsible for the final quality of products, has been investigated by a number of researchers in terms of time–temperature tolerance of frozen products (Giannakourou et al. 2006). In a real supply chain, frozen products are exposed to higher temperature and are without precise control (Taoukis et al. 1991), resulting in significant quality loss. Kinetics of quality changes as a function of time and temperature is well covered in literature; this requires regression of a quality parameter against time for given temperature range. The resulting rate of reaction, often an exponential term, is further plotted against a series of temperatures to calculate activation energy, i.e., energy required by the quality parameter to degrade. The procedure for the determination of the reaction order rate constant is described by Taoukis et al. (1997). A number of papers report the use of an Arrhenius-type relationship to determine temperature dependence of the rate of reaction, and consequently develop shelf life models in terms of chemical or microbiological reactions in foods (Tsironi et al. 2009). Zeng et al. (2005) described the quality changes of shrimp (*Pandalus borealis*) stored under different cooling conditions (1.5 to −1.5 °C) and found significant differences in sensory scores. Tsironi et al. (2009) estimated shelf life of frozen shrimp at variable temperature conditions (−5 to −15 °C) by conducting a full kinetic study and model validation. But, due to the empirical nature of the models, they are not always applicable in a wide range of products and handling conditions. Moreover, measurement of quality parameters in the time domain at subfreezing temperatures is not an easy task. The kinetic models do not take into account the likely abusive handling in the supply chain. Xie et al. (1998) has reported that for textural properties, nonlinear regression in Arrhenius arrangement is not applicable.

For physical quality parameters, such as texture and color, instrumental measuring techniques are now preferred over subjective scoring methods (Wu and Sun 2012; Pathare et al. 2013), due to their sensitive measuring ability. With huge amount of generated data in instrumental methods, it is appropriate to explore patterns in generated data to use for predictions.

To overcome modeling difficulties in food processes, evolutionary algorithms have been explored as an alternate to mathematical modeling (regression models, theoretical analysis models, or differential equations) in the past decade. Evolutionary algorithms, such as artificial neural networks and genetic algorithms, have been well documented by Enitan and Adeyemo (2011), Chen and Ramaswamy (2002), Ahmad et al. (2010), Goni et al. (2008), and Mittal and Zhang (2000).

Xie et al. (1998) compared kinetics (first order), artificial neural network (ANN), and Fuzzy Logic in modeling texture changes of dry peas. The results showed that the neural network model consistently produced the best fit to the experimental data, and the first-order-reaction kinetic model did not produce satisfactory outputs. Similarly, Yu et al. (2006) compared ANN and nonlinear regression models for predicting shrimp growth, which showed the convenience of data handling with ANNs. In short, the advantages of ANN are: (1) kinetic parameters such as reaction rate and activation energy are not required to predict loss of quality; (2) error tolerance due to noisy and incomplete data and generalization ability; and (3) ability of ANN to incorporate more than one input to model the variability of processes. It has the ability of relating the input and output parameters without any prior knowledge of the relationship between them (Chen and Ramaswamy 2002; Goni et al. 2008). ANNs may be used to estimate or predict process behavior without the need of a mathematical model or a prediction equation, associated to the physical problem (Ramesh et al. 1996).

Selection of other factors, such as learning rate, momentum term, and noise are chosen by trial and error. Ferentinose (2005) described that outputs of an ANN model heavily depend on its topology, activation function, and optimum number of neurons in hidden layers. Several strategies have been employed to optimize the network architecture, such as a trial-and-error approach, and backpropagation (BP) algorithm. The trial-and-error approach is time consuming, whereas the BP algorithm has a tendency to be stuck at local optima. In addition, the BP algorithm calculates the derivative of each weight, which makes the process complex and time consuming. To overcome these issues, optimization of ANN using genetic algorithms (GAs) (genetic algorithm–artificial neural network (GANN)) has shown promising results. GA utilizes population search approach rather than a single point and optimizes the fitness function instead of derivatives of weights (Goldberg 1989).

A number of papers have reported the use of a neural network with a genetic algorithm for process parameter optimization, such as Cook et al. (2000), Morimoto et al. (1997), Fathi et al. (2011a, b), and Izadifar and Jahromi (2007), and optimized vegetable oil hydrogenation process (Liu et al. 2007). Through the combinatorial approach of GANN, simple neural networks can be obtained with less cost and time consumption, where the goodness of fit with an error function is used to optimize network topology.

Modeling loss of quality in terms of physical parameters is an important aspect in supply chain of fresh produce. In the current available literature on frozen shrimp, no attempt has been made to model loss of quality as a function of time using evolutionary algorithms. Moreover, the enormous amount of data generated by instrumental measurements offers a considerable difficulty when analyzed with traditional statistical methods. Therefore, this study aims at evaluating performance of a GANN scheme for predicting loss of quality of frozen shrimp in terms of color and texture with

varying freezing or thawing history, storage time, and products' dimensions.

Materials and Methods

Live white shrimp (*Litopenaeus vannamei*) harvested during March 2012 in Pathum Thani province (Thailand) were procured and transferred to a laboratory in an iced box, washed thoroughly with fresh water and chlorine, sorted into lots of equal size, gutted, and deshelled before storage.

Temperature Regimes

Samples were frozen using three different laboratory grade chest freezers set at −18, −20, and −23±1 °C. In order to record temperature, the freezers (LCDF 220, Evermed, Italy) were connected with a K-type thermocouple and a data logger. A thermostat switch was used to ensure temperature maintained at a set point. After initial freezing, the samples were stored for 3 months. The samples were drawn out every 2 weeks and thawed at 35 °C for 15 min, and then cooked by immersing in hot water (60 °C) for 30 min. Cooked samples were then evaluated for quality. All readings were made in triplicate.

Quality Evaluation

Two quality indicators, namely, color and texture, were selected for the study.

Changes in color were measured as CIELab values (*L* value: lightness, a value: redness and greenness, *b* value: yellowness and blueness), by a Hunter color meter. The instrument was standardized according to the Commission International de l' Eclairage (CIE) with a standard white reference plate (calibrated as *L** 93.33, *a** −0.91, *b** 1.46). Color measurements were conducted at the end of every storage period for peeled shrimp at two points of single shrimp samples.

Changes in texture were measured using a standard compression test to peeled, squared cut, shrimp with a texture analyzer (TA-XT Stable Micro Systems, UK). A flat-ended cylinder of 20 mm diameter was selected to mimic a human finger. The fleshy part of shrimp was cut into a square with dimensions of 8×8 mm^2 in order to maintain uniformity in size (Vongsawasdi 1996; Sigurgisladottir et al. 1999; Thybo and Martens 1999). In order to construct the texture profile (Bourne 1978), the Texture Analysis software was programmed to apply double compression. The flat-ended cylinder (P35) approached the sample at a speed of 0.5 mm/s and compressed 30 % of the original height of the sample, with a holding time of 5 s. Then a second compression was made on the sample. Force–deformation curves were analyzed to calculate texture parameters. Three basic textural parameters, such as hardness (in newton)—maximum force of the first peak, cohesiveness—the ratio of force area under the first and second compression (dimensionless), and chewiness—a product of gumminess and springiness (in newton second) were obtained from the force vs. distance plot. Using texture expert system software (developed by Stable micro systems Inc. UK), a double compression was performed on the same sample with a 1-s delay in between for comparison and calculation of parameters.

Determination of Freezing and Thawing Rates

Freezing and thawing rates (FR, TR respectively) were determined (Eq. 1) according to the definition given by the International Institute of Refrigeration (1986):

$$\text{FR or TR} = \frac{T_2 - T_1}{t_2 - t_1} \tag{1}$$

where T_1 is the initial freezing temperature, T_2 is the final freezing point (−18 to −23 °C), and t_1, t_2 are the time intervals elapsed between the beginning and the end of freezing or thawing.

Determination of Shrimp Size

Dimensions of shrimp were measured in terms of width, thickness, and length. The samples were carefully peeled, beheaded, and the tail was removed before measuring using a digital calipers.

Artificial Neural Network

An ANN consisting of an input function that computes the weighted sum of the input, an activation function that transforms the weighted sum into final value is shown in Eq. 2:

$$y_j = \sum_{i=1}^{n} f\left(w_{ij} x_i\right) + b_j \tag{2}$$

where w_{ij} is the weight of the connection between neuron i and neuron j, and b_j is the bias associated with jth neuron.

A hyperbolic tangent activation function (Eq. 3) of output range of −1 and 1 was used as recommended by Jindal and Chauhan (2002):

$$\tan h\,(x) = \frac{e^x - e^x}{e^x + e^x} \tag{3}$$

The learning rule, also called gradient search, is used to calculate the weight update. As in many optimization applications *Levenberg–Marquardt NN Training Algorithm*, the

so-called LM method, is usually preferred over many other optimization techniques (Kermani et al. 1999).

Six processing elements in input layer (thickness (TH), width (*W*), length (*L*), time (*t*), FR, TR) were used. The number of neurons in hidden layer was varied from 1 to 25 depending on output error, whereas the number of layers was manually varied between 1 and 3. The output response of the network i.e., classifiers were *L**,*a**,*b** values, hardness (in newton), cohesiveness (dimensionless), and chewiness (in newton second), were used one at a time for training the network.

Genetic Algorithm

The initial population of 60 chromosomes comprising step size, weight change momentum (1, −1), learning rate, and number of neurons in hidden layer were randomly generated. Termination criterion was set when the best fitness of chromosomes was achieved or 100 generations reached, and selection of operative type was chosen as the Roulette wheel on rank basis. Uniform crossover probability of 0.9 and mutation probability was adjusted to 0.01. The ANN was trained using ranked population based on fitness. The algorithm was repeated until a solution was found that satisfied the target (mean squared error (MSE)) or the highest ranking solutions reached a plateau, and there was no further improvement with repeated iterations (Fig. 1).

Preparation of Database

Experimentally measured data points (500) were used in network development, validation, and testing. A total of 400 data rows were randomized and divided into three groups: training dataset (50 %), cross validation (c.v.) dataset (25 %), and testing dataset (25 %). Another 100 rows of dataset were reserved for production and were not used in development of the network. In order to overrule accidental bias in the dataset, the network was developed by all possible combinations of training, c.v., and testing datasets according to the arrangement shown in Table 1.

The range of minimum to maximum values of all input variable and desired output is given in Table 2. As the temperature during storage of the products was kept constant at −18 °C to mimic the actual distribution chain, it was not included as an input variable, as it did not add any useful information to the model development. The effect of rate of freezing on the quality of frozen products is also well established, i.e., faster freezing reduces the crystal formation that results in muscle damage and can have a detrimental effect on apparent color as well as eating qualities such as hardness, cohesiveness, and chewiness. A range of freezing rates, therefore, were included. Thawing and subsequent cooking are other important variables that have a direct

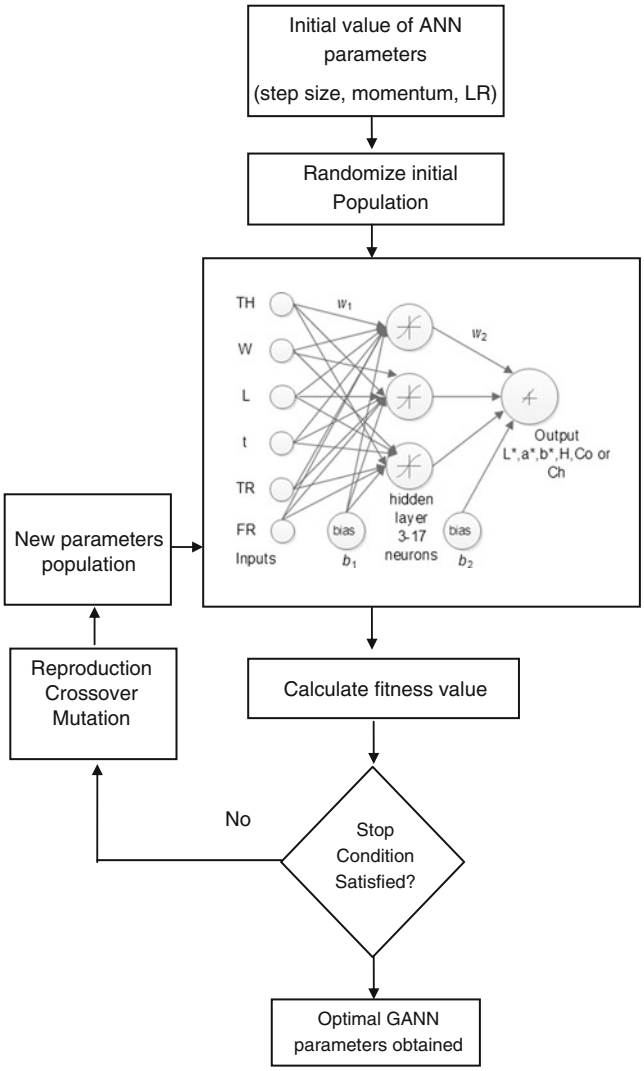

Fig. 1 Schematic diagram of optimization procedure of artificial neural network using genetic algorithm

effect on how consumers perceive quality. It should be noted that for frozen products, it is recommended that products are tested close to the state in which they are consumed. Therefore, it was necessary to uniformly thaw and cook the shrimp before measuring color and textural properties. Furthermore, the product size (thickness, width, and length) has an indirect relationship with quality indicators; Goni et al. (2008) and Mittal and Zhang (2000) used product dimensions as input variables, along with thermal properties to predict freezing time.

In order to establish relationship of input variables with the network response, sensitivity analysis of the validation set was carried out by setting all predictor values to their mean, and then network prediction was obtained. The process was repeated by setting each predictor consecutively to its minimum and maximum value (Table 2). By comparing network response of mean, minimum and maximum levels

Table 1 Arrangement of dataset for training the network (number of rows)

	Arrangement sets of data rows					
	1	2	3	4	5	6
Training dataset	1–200	101–300	201–400	1–200	101–300	201–400
C.V. dataset	201–300	301–400	101–200	301–400	1–100	1–200
Testing dataset	301–400	1–100	1–200	201–300	301–400	101–200
Initial seed value	12,345	45,678	546,783	354,761	136,573	134,276
Avg. training Error	0.063	0.049	0.045	0.031	0.06	0.053

of predictors, an indicative effect of input variables was explored. Finally, the product sum of connecting weights was used to determine the significance of input variables as depicted in Table 6, Section "Results and Discussion".

Evaluation Criterion

Performance of network was evaluated by the average error and the MSE in training, validation, and test datasets. Definition of both indicators is given below (Eqs. 4 and 5):

$$\mathrm{MSE} = \frac{\sum_{j=0}^{P}\sum_{i=0}^{N}\left(d_{ij}-y_{ij}\right)^2}{N\,P} \tag{4}$$

and

$$\text{Average error} = \frac{\mathrm{SSE}}{n} \tag{5}$$

The average error depicts the average sum of squares of residuals in the model (training dataset in this case) based on total sample size. In order to establish correlation between experimental values and network predicted values, the coefficient of correlation (R^2) and root mean squared error (RMSE) were employed (Eqs. 6, 7):

$$R^2 = 1-\frac{\left(y_i-\overline{y}\right)^2}{\left(y_i-e_i\right)} \tag{6}$$

and

$$\mathrm{RMSE} = \frac{1}{N}\sqrt{\left(y_{\text{pred.}}-y_{\text{expt.}}\right)^2} \tag{7}$$

Table 2 Range of input and output variables used for training NNs (n=400)

	Minimum	Maximum	Mean
Input variables			
Freezing rate (°C min^{-1}), FR	0.1	0.6	0.35
Thawing rate (°C min^{-1}), TR	3.9	5.8	4.85
Storage time (days), t	12	90	51
Sample thickness (m), TH	0.01	0.019	0.0145
Sample width (m), W	0.008	0.015	0.012
Sample length (m), L	0.1202	0.154	0.1371
Output variables			
L^*	37.89	61.655	49.7725
a^*	0.32	3.03	1.675
b^*	17.07	27.4	22.235
Hardness (N)	17.37	95.2	56.285
Cohesiveness (dimensionless)	0.054	3.94	1.997
Chewiness (N s)	10.66	261.77	136.215

Data Analysis

Statistical analysis of the results was performed using the ANOVA (SPSS ver. 12 software package). The statistical significance of multiple comparisons was evaluated by Duncan's significant difference test at 95 % confidence level ($p<0.05$).

Results and Discussion

Initially, the neural network was trained by randomly initializing training parameters to ascertain the effect of number of hidden layers and nodes in each layer. For one to three hidden layers, the nodes in each layer were varied as 5, 15, and 25. The learning rate and momentum was fixed at 0.5. It was observed that average training error decreased as the number of hidden layer and nodes in each layers increased (data not shown). However, no further change in training error was observed when more than three hidden layers were used. It is particularly important that a fewer hidden layers with optimum number of nodes are used to make the network powerful enough to learn the data pattern and generalize without lengthy computations, as noted by Jindal and Chauhan (2002). The number of hidden layers and nodes were, therefore, fixed at a previously determined level as those heavily depend on the nature of the problem.

In order to utilize GA capability to optimize network topology, individual haploid chromosomes consisting of three genes were represented by weight change momentum (1,−1), learning rate, and number of neurons in hidden layer

Table 3 GA optimal parameters for training ANN for the prediction of color and texture quality

GA model parameters	L^*	a^*	b^*	H	Co	Ch
No. of generations	10	39	9	15	100	51
Operator Type (P=0.9)	xover	xover	xover	xover	xover	xover
Training time (min)	4.2	16.3	3.8	6.3	41.7	21.3
Fitness threshold	0.001	0.001	0.001	0.012	0.1	0.014

(<25). The chromosomes were allowed to reproduce to a maximum of 100 generations with a fitness threshold criterion of 0.001. The parameters of reproduction i.e., uniform crossover probability, mutation probability, and elimination proportion were 0.9, 0.01, and [0 1], respectively.

Tables 3 and 4 depict the GA parameters for NN optimization; for the data with linear tendency, up to 10 generations were sufficient to converge to the threshold fitness, as in the case of L^* and b^* values. However, for other quality parameters, if the fitness threshold was not achieved up to 100 generations, optimization was stopped to avoid over fitting of desired outputs. Typically, a weight change momentum of 0.6 and a learning rate of 0.04 were attained.

For color parameters, the a^* value took longest time of 16.3 min to reach optimum points with 39 generations and 1,000 epochs, followed by L^* and b^* values. Similarly, simulation time of 41.7, 21.3, and 6.3 min were recorded for cohesiveness, chewiness, and hardness, respectively. It was obvious that for more generations, a longer time was required for optimization (Table 3). The prediction of cohesiveness was found to be less accurate as compared to hardness and chewiness. This confirms the findings an earlier study (Monaco et al. 2007) in which both linear regression and partial least square analysis yielded the lowest prediction ability relative to other textural variables.

An ANN trained using 2–25 neurons in a hidden layer with the GA yielded the optimum configuration of the network, which gave the lowest prediction error with average error ranging from 0.01 to 0.15, MSE (validation dataset) ranging from 0.01 to 0.082, and MSE (test dataset) from 0.061 to 0.55, for prediction of color parameters (L^*,a^*,b^*) and textural properties (hardness, cohesiveness, and chewiness). Optimum values of neurons in hidden layer, learning rate, and momentum are shown in Table 4.

The MSE of the training dataset was a little lower than the MSE of the validation dataset, which implied the networks' generalization ability. In order to further validate the models, a third group of dataset (test dataset) was used to generate desired output values, and it was evaluated in terms of RMSE and R^2 values for the experimental versus predicted values. A low RMSE in the range of 0.5–1.6 and correlation coefficient of more than 0.9 in all cases were obtained (Table 5).

In addition, for the six processing elements (neurons) in an input layer (TH, W, L, t, FR, and TR) the weight matrix (w_1) between input and hidden layers was obtained as 3×6, 5×6, 10×6, 17×6, 15×6, and 8×6 for TH, W, L, t, FR, and TR, respectively. The weight matrix (w_2) between the hidden layer and the output layer was found to be 6×1 for all output variables. The values of connecting weights and the corresponding biases (b_1,b_2) are shown in the Appendix.

Significance of Input Variables

In order to explain contribution of each of the variables for the prediction of a dependent variable, relative importance of input variables was determined by the product sum of connecting weights of the network, as suggested by Mittal and Zhang (2000) and Olden and Jackson (2002). It was demonstrated that by using Garson's algorithm (Garson 1991) for partitioning and quantifying neural network connection weights, the relationship with predictor-dependent variables can be explained by the larger the sum for a given input node, the greater the importance of the corresponding input variable. The relative importance (R_I) of input variables is defined by:

$$R_I = \sum_{Hi=I}^{h} (w_{IHi} * w_{HiO}) \tag{8}$$

where R_I is the relative importance of the input variable I, h is the total number of hidden nodes, w_{IHi} is the weight connection between input node and hidden node Hi, and w_{HiO} is the weight connection between hidden node Hi and

Table 4 The optimal ANN training parameters

ANN training parameters	L^*	a^*	b^*	H	Co	Ch
No. of hidden layers	1	1	1	1	1	1
No. of nodes in hidden layer	3	5	10	17	15	8
Weight change momentum (−1,1)	0.7	0.6	0.6	0.6	0.6	0.6
Learning rate	0.04	0.04	0.04	0.04	0.04	0.04

Table 5 Evaluation of the GANN model for the prediction of color and texture quality parameters

Model GANN	L^*	a^*	b^*	H	Co	Ch
MSE (training dataset)	0.003	0.022	0.018	0.012	0.021	0.085
MSE (c.v. dataset, n=50)	0.022	0.191	0.082	0.049	0.202	0.518
RMSE (test dataset, n=50)	0.65	1.6	0.557	1.654	0.11	1.557
R^2 (actual vs. predicted) c.v. dataset	0.957	0.91	0.992	0.95	0.87	0.89

output node. In this approach, connection weights obtained in all trainings were used as average values at the GA-optimized initial weight change momentum (Table 3). Contribution of each input neuron to the output through hidden neurons was calculated as the product of input–hidden and hidden–output connections. The final sum of all connections was converted into relative terms for comparison purpose. By comparing the relative importance of input variables to the ANN-predicted output, the ability of ANNs of drawing inference in providing an explanation of how the input variables have contributed for the prediction of dependent variable was established, analogous to regression techniques. It was found that freezing and thawing rates (FR and TR) and storage t had contributed more than the product dimensions, especially for L^* and textural properties, except chewiness (Table 6).

The L^* value (lightness) decreased as the storage period increased. The samples drawn during first half of the storage period of less than 45 days had an apparent luminance which started to disappear, and products appeared less shiny. In contrast, the yellowness, b^*, value increased over time. The samples drawn at the later stage of storage were redder, as compared to the initial stage. The results were consistent with the findings reported by Tsironi et al. (2009), who reported color degradation during frozen storage of shrimp modeled by a zero-order relationship. The network topology of one hidden layer with 3–10 nodes for the prediction of L^* a^* b^* values represented a simple noncomplex solution. The MSE of validation dataset was higher than the training dataset, but within acceptable range, for all the color values, signifying the stability of the network. The prediction error in the test dataset was, however, higher in the case of L^* and b^* values (Table 3).

As the storage time increased, the samples "mouth feel" deteriorated, which is related with the decrease in hardness (Erdoğdu and Balabana 2000) due to thermal softening. Other parameters such as cohesiveness and chewiness give additional information on sensorial qualities. As can be seen in Table 4, simple neurons with one hidden layer were sufficient to predict textural properties. Only in the case of cohesiveness, noise in prediction was higher than the other textural properties with RMSE 1.6. Other studies have reported an R^2 of 0.7–0.9 for the prediction of hardness through multiple linear regressions (Luzuriaga et al. 1997). Monaco et al. (2007) found high correlation between sensory and instrumental hardness measurement (R^2=0.87), but cohesiveness did not show satisfactory relationship.

Weight Matrices and Working Equations

The network response (NR) to actual input values (IV) for each quality parameter is given by a general equation (Eq. 9) where IV represent a column vector, and w_1 and w_2 , which are the weight matrices between input layer–hidden layer and hidden layer–output layers, respectively, represent equation constants. Also, b_1 and b_2 are the corresponding *bias* terms and f_1 and f_2 are referred to as transfer functions:

$$\mathrm{NR} = f_2\,[w_2\ f_1\{w_1 IV + b_1\}] + b_2 \tag{9}$$

With the help of a computer program, the optimized network can be implemented using the network constants (weights and biases), provided in the Appendix.

Table 6 Relative significance of input variables to the dependent variables (%)

Input Variables	Output variables					
	L^*	a^*	b^*	Hardness	Cohesiveness	Chewiness
Thickness (TH)	8.31	22.30	16.89	10.46	0.59	5.21
Width (W)	9.96	15.08	11.85	8.87	14.70	19.51
Length (L)	2.16	4.29	18.89	16.39	21.45	21.12
Time (t)	29.47	14.40	16.00	15.14	15.41	26.63
Freezing rate (FR)	15.84	27.00	17.84	14.43	16.37	4.35
Thawing rate (TR)	34.25	16.93	18.52	34.70	31.48	23.18

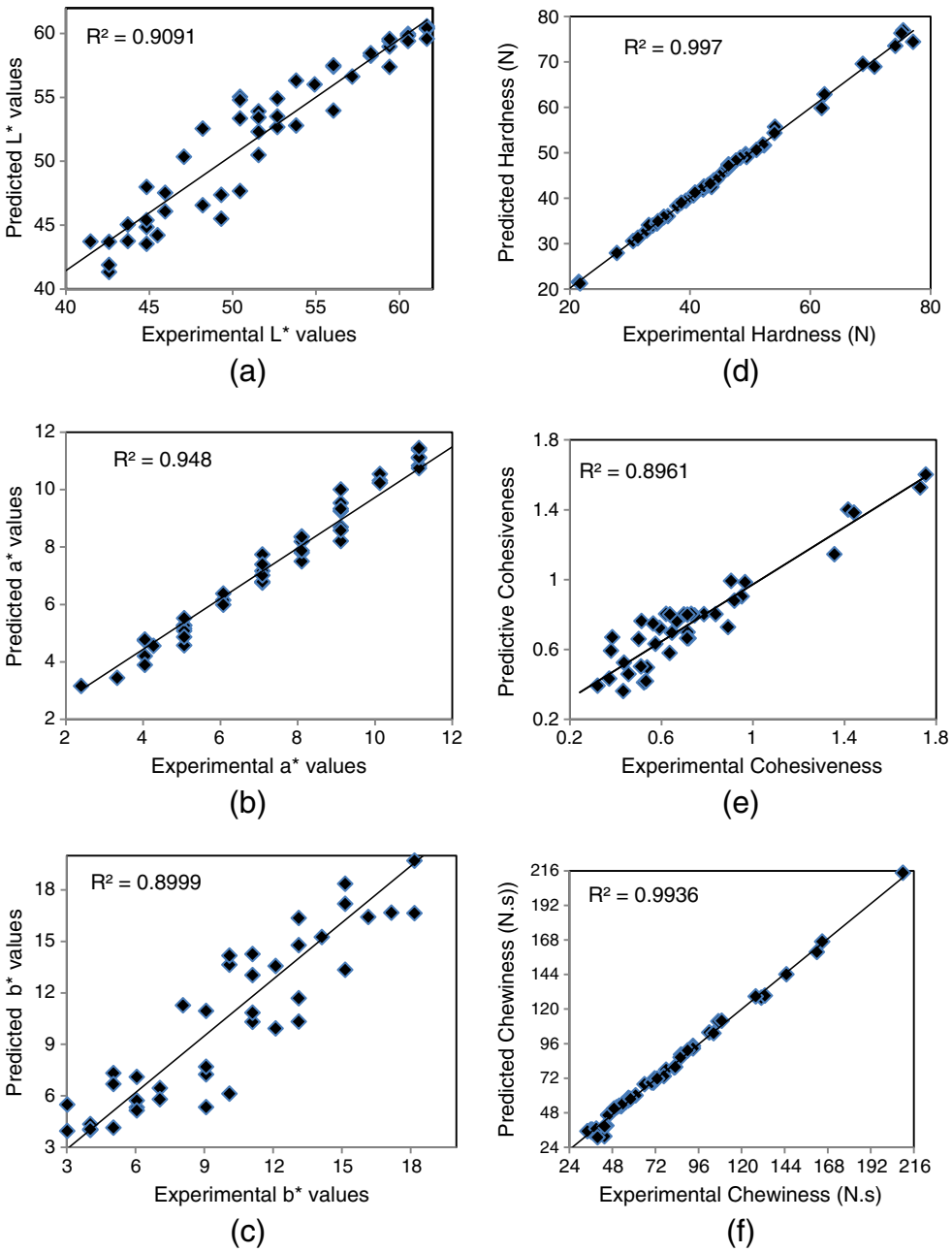

Fig. 2 Comparison of GA-optimized network predicted values and experimental values of **a** L^*, **b** a^*, **c** b^*, **d** hardness, **e** cohesiveness, and **f** chewiness

Table 7 Comparison of MSE calculated by MLR, BP, and GANN

Quality parameter	MLR†	BP††	GANN†††[a]
L^*	1.502±0.12	0.24±0.026	$0.022 \pm 1\times10^{-3}$
a^*	0.53±0.025	0.124±0.026	$0.191 \pm 9.5\times10^{-4}$
b^*	1.46±0.05	0.193±0.0016	$0.082\pm4.1\times10^{-3}$
Hardness	4.7±0.16	0.22±0.01	$0.049\pm2.4\times10^{-3}$
Cohesiveness	0.431±0.18	0.15±0.0058	$0.202\pm1\times10^{-3}$
Chewiness	2.6±0.06	0.25±0.005	$0.518\pm2.5\times10^{-2}$

Note: Different number of † indicate significant difference ($p<0.05$) by Duncan's test

[a]MSE obtained in c.v. dataset was used

Evaluation of the Network

The GANN models were evaluated for their correctness and generalization ability, to establish that these developed models can be employed in real world situation. An unseen dataset ($n=50$) was plotted against network predicted values. Figure 2 (a–f) shows the relationships between experimental and predicted values through GANN models. High correlations ($R^2=>0.9$) were found with slope of X-Y regression line close to 1. It can be seen that the color parameter a^*, texture parameters, hardness, and chewiness data closely follow the regression line, while L^*, b^*, and cohesiveness show considerable scatter. This may imply that for these variables, the optimized network did not perform well with the dataset not shown to the network during its training, and thus could limit its generalization ability. Nevertheless, these results show that instrumental measurements of color and texture can be estimated through product dimensions, rates of freezing and thawing, and time it spends in distribution.

Comparison of ANN Models with Multiple Linear Regression

For comparison purposes, multiple linear regression (MLR) and backpropagation ANN were used to predict the quality parameters. Based on MSE of prediction, it was observed that a GA-optimized Neural Network performed better than MLR and BP (Table 7). The prediction errors were significantly different with 95 % confidence level using Duncan's multiple range test. It can be clearly seen that ANN models outclassed the MLR for a multifaceted set of data generated at a commercial handling environment of frozen shrimps. These results have been found consistent with the study of Yu et al. (2006) for the prediction of shrimp growth using ANN, in comparison with regression models.

Although, in general, ANN produced a better prediction of physical quality changes in frozen shrimp than MLR, the lowest prediction error was obtained by a GA-optimized ANN, considering the inherent variability in estimation of the color and textural quality of frozen shrimps. In order to avoid the impending tendency of BP algorithms to over fit a correlation model, GANN offers an alternative, efficient, and simple way of more accurate prediction.

Conclusion

The results showed that selected input variables (FR, TR, t, TH, W, and L) could successfully be used to predict color and textural parameters of frozen shrimp. As for textural properties, in contrast to an Arrhenius-type relationship, GANN offer a robust intelligent solution. Textural properties of sensorial importance (cohesiveness and chewiness) can be predicted from the product handling history. Although, the color degradation can be explained with a zero- or first-order kinetic model, application of GANN is a step towards machine learning and inline implementation of color measuring system. However, a neural network should be employed carefully to avoid their inherent drawback by performing a sensitivity analysis and using the sum product of connecting weights to have a clear understanding of relationships that exist between the input variables and the output (desired) variables. The GA optimization technique yielded satisfactory network topology and training parameters, such as learning rate, weight change momentum, and number of neurons in the hidden layer. The reproduction of NN parameters using GA features, such as mutation and crossover, reduces the possibility of trapping the solution at local optima. Unlike MLR and BP, GANN predicted the desired outputs with high accuracy and lower error. Different error criteria such as MSE and RMSE for training, cross validation, and test datasets confirmed the network stability. In conclusion, ANN is a useful tool to handle large data for meaningful conclusion; accuracy of prediction may be increased if it is combined with GA. The traditional modeling technique to estimate activation energy of the deteriorating reaction and equation constants, which makes the solution cumbersome and is not robust to apply, can be replaced with the proposed GANN scheme. GANN, however, requires a significant time to optimize the network depending on number of generations in which the chromosomes mate to reach a stopping criterion. A further attempt should be made to improve the accuracy of estimation by applying unsupervised ANN for determining the presence of possible clusters in the raw data.

Appendix

Connecting weight matrices

1. Input variables: TH, W, L, t, FR, TR; Output variable: L^*

$$w_1 = \begin{bmatrix} 5.63 & 0.02 & 0.91 & 0.26 & 0.05 & 3.2 \\ 2.48 & -1.18 & 4.45 & -2.9 & -2.96 & -7.3 \\ 0.02 & 0.11 & 0.46 & 0.14 & 0.04 & 0.03 \end{bmatrix}$$

$$w_2 = \begin{bmatrix} 0.075 \\ 0.067 \\ 0.0018 \end{bmatrix} \quad b_1 = \begin{bmatrix} 0.075 \\ -0.067 \\ 0.0018 \end{bmatrix} \quad b_2 = [0.001]$$

2. Input variables: TH, W, L, t, FR, TR; Output variable: a^*

$$w_1 = \begin{bmatrix} 0.011 & 0.13 & 0.15 & 2.56 & 8.3 & 0.32 \\ 1.68 & 0.90 & 0.0045 & -0.14 & -2.96 & 0.2 \\ 0.22 & 0.25 & 3.1 & 1.25 & 0.20 & 0.49 \\ 4.30 & 0.50 & 1.40 & -0.02 & 1.23 & 3.10 \\ 0.15 & 4.50 & 0.29 & 0.42 & 0.043 & 6.80 \end{bmatrix} w_2 = \begin{bmatrix} 7.2 \\ 1.3 \\ -0.638 \\ 8.5 \\ 0.009 \end{bmatrix} b_1 = \begin{bmatrix} 0.005 \\ 0.007 \\ 0.001 \\ -0.002 \\ 0.00013 \end{bmatrix} b_2 = [0.0005]$$

3. Input variables: TH, *W*, *L*, *t*, FR, TR; Output variable: *b**

$$w_1 = \begin{bmatrix} 5.63 & 0.02 & 0.05 & 0.91 & 3.2 & 0.62 \\ 2.48 & -1.18 & 4.45 & -2.9 & -2.96 & -7.3 \\ 0.02 & 0.11 & 0.46 & 0.14 & 0.04 & 0.39 \\ 0.2 & 0.01 & 1.30 & -0.05 & 0.23 & -0.04 \\ 0.04 & 0.50 & 0.05 & 0.22 & 0.43 & 0.01 \\ 0.011 & 0.13 & 0.17 & 8.3 & 0.015 & 0.01 \\ 1.68 & 0.9 & 0.0045 & -0.14 & 0.02 & 0.15 \\ 0.22 & 0.29 & 0.25 & 3.1 & 0.24 & 0.2 \\ 4.3 & 0.29 & 4.5 & 0.42 & 3.1 & 6.8 \end{bmatrix} w_2 = \begin{bmatrix} 0.04 \\ -0.14 \\ 0.05 \\ 8.3 \\ 0.02 \\ 0.24 \\ 1 \\ 3.1 \\ -0.02 \\ 0.25 \end{bmatrix} b_1 = \begin{bmatrix} 0.035 \\ 0.0003 \\ -0.075 \\ 0.001 \\ 0.0028 \\ -0.00103 \\ 0.00018 \\ 0.0011 \\ 0.067 \\ 0.0013 \end{bmatrix} b_2 = [0.0015]$$

4. Input variables: TH, *W*, *L*, *t*, FR, TR; Output variable: H

$$w_1 = \begin{bmatrix} -7.8 & 0.02 & 5.13 & 0.26 & 0.52 & 0.02 \\ -2.18 & -1.38 & 1.45 & -1.9 & -2.16 & 1.2 \\ -3.2 & 0.18 & 0.36 & 0.34 & 1.04 & 0.09 \\ 0.04 & 0.05 & 0.30 & -0.25 & -0.14 & -0.01 \\ 0.011 & 0.130 & 1.3 & 0.015 & 0.43 & 1.56 \\ 1.67 & 0.92 & 0.37 & 6.13 & 2.9 & 0.15 \\ 1.18 & 0.93 & 0.45 & 0.14 & -0.52 & 3.13 \\ 0.52 & 0.09 & 1.25 & -1.1 & 0.17 & 6.8 \\ 0.12 & 0.19 & 0.29 & 0.72 & 0.1 & 3.08 \\ 0.11 & 0.07 & -0.9 & -1.4 & 0.13 & 0.06 \\ -0.013 & 0.5 & -0.012 & 1.23 & 3.1 & -0.8 \\ 0.19 & -1.2 & 1.1 & 2.5 & 0.014 & 0.77 \\ -1.3 & 0.015 & 2.2 & 0.15 & 1.1 & 1.36 \\ 1.9 & -0.12 & 0.15 & 0.01 & 0.29 & 0.2 \\ 0.008 & -1.4 & 0.1 & -0.8 & -0.7 & -2.6 \\ 0.06 & -1.17 & 0.13 & 2.48 & -1.18 & 4.45 \\ 1.1 & -0.008 & -0.18 & -0.6 & 2.3 & 1.4 \end{bmatrix} w_2 = \begin{bmatrix} 0.023 \\ 0.0975 \\ 3.1 \\ 0.7 \\ -0.15 \\ 8.3 \\ 1.09 \\ 0.05 \\ -0.17 \\ 0.5 \\ 1.8 \\ -0.69 \\ -0.009 \\ 2.3 \\ -0.75 \\ 0.007 \\ 0.6 \end{bmatrix} b_1 = \begin{bmatrix} 0.00023 \\ -0.003 \\ -0.007 \\ 0.00015 \\ -0.0001 \\ 0.001 \\ 0.075 \\ 0.00013 \\ 0.067 \\ 0.002 \\ 0.00005 \\ -0.0014 \\ 0.00017 \\ 0.0018 \\ -0.0014 \\ 0.0002 \end{bmatrix} b_2 = [0.0018]$$

5. Input variables: TH, *W*, *L*, *t*, FR, TR; Output variable: Co

$$w_1 = \begin{bmatrix} 1.45 & 0.02 & 0.91 & -0.7 & -0.8 & -0.26 \\ 2.48 & -1.18 & 1.45 & -1.3 & -2.96 & -1.9 \\ -7.8 & 0.11 & 0.46 & 0.14 & 0.04 & 0.03 \\ -0.2 & 2.05 & 1.30 & -0.5 & -0.14 & -0.04 \\ 0.04 & 0.05 & 0.05 & 0.22 & 0.03 & 0.01 \\ 0.01 & 0.13 & 0.01 & 0.42 & 3.1 & 6.8 \\ 1.68 & 0.9 & 0.45 & -0.14 & 2.9 & 0.5 \\ 0.22 & 0.29 & 0.25 & 3.1 & 0.27 & 0.2 \\ 4.3 & 0.5 & 1.29 & 0.02 & 0.12 & 3.1 \\ 0.15 & 4.5 & 0.9 & 0.5 & 0.3 & 1.3 \\ -0.13 & 0.09 & -0.32 & -1.43 & 5.1 & -0.06 \\ 0.11 & -0.01 & 1.15 & 0.5 & -0.013 & -0.8 \\ -1.3 & 0.012 & 2.2 & -0.14 & 0.009 & 0.77 \\ 1.3 & -0.15 & -0.15 & 0.45 & 0.014 & 1.36 \\ 0.18 & -1.4 & 0.1 & 3.2 & -0.26 & -0.06 \end{bmatrix} w_2 = \begin{bmatrix} 0.03 \\ 3.1 \\ 0.014 \\ 0.7 \\ 3.6 \\ 8.3 \\ 0.02 \\ 0.25 \\ -0.32 \\ 1.6 \\ -0.36 \\ 1.8 \\ -0.69 \\ 1.15 \\ 2.3 \end{bmatrix} b_1 = \begin{bmatrix} 0.0013 \\ 0.001 \\ -0.0705 \\ 0.0016 \\ -0.00014 \\ 0.0025 \\ 0.0032 \\ 0.067 \\ 0.0015 \\ 0.0001 \\ -0.0009 \\ -0.0018 \\ -0.001 \\ 0.0018 \\ 0.0021 \end{bmatrix} b_2 = [-0.0035]$$

6. Input variables: TH, *W*, *L*, *t*, FR, TR; Output variable: Ch

$$w_1 = \begin{bmatrix} 1.8 & 0.12 & 0.9 & -0.8 & 0.52 & 0.02 \\ 0.2 & -1.17 & 0.035 & -1.9 & 1.3 & 1.2 \\ 0.04 & 0.11 & -0.46 & 0.34 & 0.04 & 0.09 \\ -0.26 & 0.65 & 1.30 & -0.22 & -0.5 & -0.01 \\ -1.2 & 0.1 & 1.08 & 0.015 & 0.43 & 1.56 \\ 0.03 & 1.45 & 0.47 & 6.13 & 2.9 & 0.15 \\ -0.04 & 1.48 & 0.5 & 0.14 & -0.52 & 3.13 \\ 0.022 & 0.42 & -0.1 & -0.8 & -0.7 & -2.6 \end{bmatrix} \quad w_2 = \begin{bmatrix} 0.023 \\ 1.09 \\ 0.05 \\ -0.17 \\ 0.5 \\ 1.8 \\ -0.009 \\ -1.8 \end{bmatrix} \quad b_1 = \begin{bmatrix} 0.0013 \\ 0.001 \\ -0.075 \\ 0.00015 \\ -0.067 \\ 0.0001 \\ 0.0012 \\ -0.0018 \end{bmatrix} \quad b_2 = [0.0008]$$

TH	Thickness
W	Width
L	Length
t	time
FR	Freezing rate
TR	Thawing rate
*L*a*b**	CIE color parameters
H	Hardness
Co	Cohesiveness
Ch	Chewiness

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International Food Research Journal 20(4): 1833-1842 (2013)
Journal homepage: *http://www.ifrj.upm.edu.my*

Characterization of quality degradation during chilled shrimp (*Litopenaeus vannamei*) supply chain

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Article history

Received: 25 October 2012
Received in revised form: 25 February 2013
Accepted: 5 March 2013

Keywords

Shrimp
Chilled chain
Quality loss
Texture
Sensory index
Shelf life

Abstract

Loss of quality in white shrimps (*Litopenaeus vannamei*) during a cold supply chain (0-8°C for 96 hours) was measured and modeled at constant and variable temperature conditions. Quality parameters such as color, Texture Profile Analysis (TPA), Total Viable Count (TVC), Total Non-Volatile Basic Nitrogen (TVB-N), and Sensory Index (SI) were selected as indices of quality. A bulk mean temperature (T_{bm}) function was calculated to describe the effect of storage time and temperature, to which the shrimp were subjected, on the quality indices using stepwise multiple linear regressions (R^2 = 0.88-0.99, SEE = 0.045-15.63). Color degradation (L^*, a^*, b^* index), SI, and TVC (CFU/g) in cold chain were adequately described by a zero order reaction with R^2 values > 0.9. In order to reduce the number of variables obtained in TPA, hardness (H), springiness (S), gumminess (G), cohesiveness (Co) and chewiness (Ch), were subjected to multiple linear regression. The hardness, H, turned out to be the most significant parameter ($P < 0.05$). Using the Arrhenius equation kinetic parameters, reaction rates (k, $min.^{-1}$) and activation energy (E_a, KJ. $Mole^{-1}.^oK$) were calculated for all the textural properties. The developed models were used to estimate remaining shelf life in terms of H, TVC, TVB-N and SI at 4 different distribution stages of a supply chain. The predicted values showed a good relationship ($R^2 \geq 0.9$) with the experimental data for all the quality parameters. The correlation matrix of the dependent variables showed that color parameters (L^*,b^*), pH, and sensory index were positively correlated ($P < 0.05$) with H, TVC, and TVB-N, respectively. However, after 84 hours of storage in variable temperature conditions, the level of TVB-N was still within the acceptable range (≤ 25 mg/100 gm N), but samples were unacceptable due to high microbial growth (> 7.5).

Introduction

Variability in temperature during storage and handling determines quality loss rates and the final level of quality of perishable foods. Seafood, such as shrimp, is highly susceptible to postharvest spoilage, and therefore, handled at low temperatures. Unlike the frozen shrimp market, which mainly serves premium local and export markets, a cold supply chain serves domestic markets. Although, the shrimps are caught and transported alive (submerged in iced water < 5°C), death occurs during transportation from farm to retailers, inducing postmortem changes in quality parameters. Shrimps are put on display for 12-48 hours and then, optionally, spend another 24-48 hours in household refrigerators before consumption.

Various chemical and physical changes take place after harvesting and during storage. From a consumer point of view, color fading (Chandrasekaran, 1994), and odor and textural changes (Tsironi *et al.*, 2009) are the most important quality parameters. These quality losses are due to complex physico-chemical reactions such as lipid oxidation, de-naturation of protein, sublimation, and re-crystallization of ice. A detailed account of chemical changes (lipid oxidation, volatile basic nitrogen) in frozen shrimp quality has been reported by Riaz *et al.* (1990). Tsironi *et al.* (2009) modeled shelf life of frozen shrimp in variable temperature conditions. They included color, texture, pH, microbial quality, and T-VBN as the indices of quality. The rate of quality deterioration in time and temperature domain was expressed by the Arrhenius equation (E_a = 118 to 156 kJ/mol). Yamagata and Low (1995) measured quality loss in terms of formaldyhyde changes in iced shrimp and recommended 4 days of shelf-life. However, complex biochemical changes cannot be easily related with consumer's perception of quality (Sloof *et al.*, 1996). Nevertheless, both physical and chemical quality indicators have been used to quantify shrimp freshness. Zeng *et al.* (2005) compared cold water shrimp (*Pandalus borealis*) stored under chilled (1.5°C) and super chilled (-1.5°C)

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conditions (6 days of storage) in terms of microbial growth, TVC, pH, and TVB-N.

Quality parameters

Color is a parameter of foremost importance, measured in terms of Hunter color parameters (L^*, a^*, and b^*), and the total change in color (ΔE) (Ahmed and Shivhare, 2001). Translucent appearance of fresh shrimp turns darker with time, which is quantified in terms of the b^*-value (yellowness) and L^*-value (lightness), during shrimp storage. Color is measured at the time of harvest, prior to cooking, and after cooking to determine the change. The change in color is dependent on sample composition and storage temperature. A zero order relationship has been used to describe the change in b-value over a period of time (Tsironi *et al.*, 2009).

Texture has been attributed as one of the most important quality factor for a product's acceptability (Monaco *et al.*, 2007) and is defined as an expression of structural, mechanical and surface attributes detected through human senses (Szczesniak, 2002). Texture depends on a sample's physico-chemical properties and human perception. Various eating preferences have been identified in developing textural profile analysis of solid foods. Among them, hardness, cohesiveness, and springiness are the most relevant to sea foods. Hardness is estimated during the first mastication, i.e., force is applied on the food product, resulting in a linear curve until the first peak, which represents a sample's strength. Other parameters derived from the curve are described by Bourn (1978).

pH is an indication of acidity, which is also related with the growth of microbes in the samples. The activity of microorganisms is the prime cause of spoilage in fresh seafood since they give rise to undesirable flavors and odors. Total Viable Count (TVC) is used as the acceptability index in standards, guidelines and specifications (Qingzhu *et al*., 2003). Total Volatile Basic Nitrogen (TVB-N) is an indicator of seafood freshness primarily due to spoilage bacteria (Huss, 1995). However, this quality indicator cannot be attributed to solely microbial spoilage due to the fact that other contributing factors, such as autolytic enzyme reactions do exist.

Sensory evaluation is conducted to complement chemical and physical methods. This determines the degree of reliance on objective (instrumental) methods. A sensory index has been developed for fresh shrimps, based on appearance, feel (texture), and color (luster) of shrimp used by buyers and inspectors in the market (Sveinsdottir *et al*., 2003). Zeng *et al*. (2005) has divided sensory quality characteristics into 5 classes ranging from excellent to spoiled. Inter-relationships with sensory and instrumental tests have been reported for cooked shrimp texture, color, and general appearance (Giménez *et al.*, 2012).

Although, a number of papers have been published on modeling shelf life quality in frozen shrimp (Nielsen and Jorqensen, 2004; Tsironi *et al*., 2009), modeling loss of quality in chilled shrimp from the perspective of supply chain management has not been given due attention due to the fact that fresh shrimps are mostly traded in conventional wet markets where modern quality assurance requirements are not in place. However, there is an increasing demand for monitoring, controlling, and recording critical parameters throughout a product's life cycle, i.e., from production to consumption. Among other factors, temperature during handling and storage is the most important factor that directly determines shelf life of a product. In actual practice, temperature during handling and storage vary from the recommended temperature range in a cold distribution chain (Giannakourou *et al.*, 2006). With modern temperature measuring and recording devices such as RFID (Radio Frequency Identification) temperature sensors (Jedermann *et al*., 2009), cellular and Zig-Bee based sensors (Ruiz-Garcia *et al.*, 2008), it is now possible to wirelessly measure and record temperature in almost all the stages of supply chain as an integrated traceability system (Ruiz-Garcia and Lunadei, 2010). The data generated through a monitoring system can be used to develop empirical quality models and to establish temperature dependency of most of the quality deteriorating reactions. In addition, empirical models are needed for developing quality assignment models representing a homogeneous group of consumers for their quality perception (Sloof *et al*., 1996). To make use of the collected data of time and temperature, quantitative relationships between storage conditions and shelf life are relevant to the current market need (Giannakourou *et al*., 2006). Information of shrimp quality loss patterns will help in developing inventory replenishment models based on actual quality (Taoukis, 1999).

Therefore, the objective of this study was to characterize the quality deterioration behavior of shrimp (*Litopenaeus vannamei*) stored and handled at low temperatures (0-8^{o}C), specifically, to study the changes in physical, chemical, and microbial indices of spoilage at constant and variable storage temperatures.

Materials and Methods

Live white shrimp (*Litopenaeus vannamei*) were captured from a local fresh water farm located in Pathum Thani province (Thailand) during March 2012. Samples were transported to a laboratory in iced box, washed thoroughly with fresh water and chlorine, and sorted to have equal size, and stored.

Two different storage scenarios were used: (i) fixed storage temperatures, i.e., 0, 3, 5, and 8°C for monitoring changes in quality and, (ii) a single lot undergoing fluctuating storage temperature in the range of 0-8°C to mimic the actual commercial handling. For the first scenario, separate commercial refrigerators (SANYO SR-F415) were used and for the latter, specially designed cooling chambers were used to store shrimps (Figure 1). The cooling chamber was consisted of 3 identical containers to collect data in triplicate. Each container was coiled with duct containing cooling media (Freon) through a compressor. A K-type thermocouple was used, to insert in 3 different positions in the container, to record temperature output per second with the help of a digital temperature controller and a data logger. The time-temperature controller was programmed to resemble the real supply chain temperature scenario. After placing the samples inside the chamber, air from inside the chamber was pumped out using vacuum pump to avoid initial quality loss due to the presence of oxygen. The temperature was varied between 0–8°C over a period of 4 days (Figure 2). There was no fluctuation. The resolution of measuring equipment was 0.1°C. According to manufacturer's specification there was an accuracy tolerance of 0.01%+0.03°C of reading (Center 309 Temperature controller and Data Logger, Center Technology Group, Taipei, Taiwan). The samples were collected every 12 hours for the quality analysis.

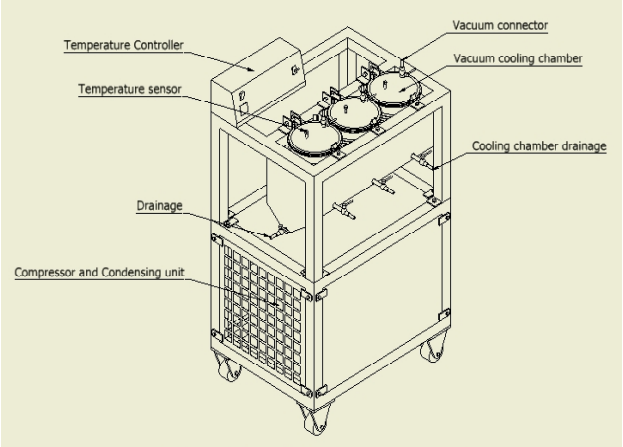


Figure 1. A schematic diagram of cooling chambers to simulate temperature fluctuation in a cold supply chain

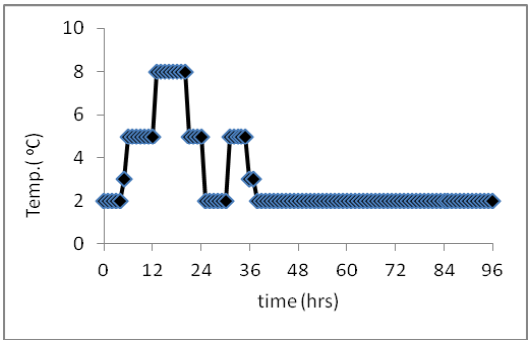


Figure 2. Typical temperature profile of fresh shrimp from harvesting, to retail and household storage.

Determination of quality parameters

Color measurement

Change in color was measured as CIELab values (L^*-value: lightness, a^*-value: redness and greenness, b^*-value: yellowness and blueness), by a Hunter Color meter. The instrument was standardized according to the CIE (Commission International de l' Eclairage) with a standard white reference plate (calibrated as L^* 93.33, a^* -0.91, b^* 1.46). Color measurements were conducted at the end of every storage period at two points of peeled single shrimp samples. The average L^*, a^* and b^* values were converted into total change in color (ΔE), according to the following formulae (Eq. 1):

$$\Delta E = \sqrt{(L_t - L_0)^2 + (a_t - a_0)^2 + (b_t - b_0)^2} \quad \text{Eq 1}$$

where L_0, a_0, and b_0 are the initial color values when storage time was zero. The corresponding L_t, a_t, and b_t shows the color values at a given storage period (in hours).

Texture measurement

Change in texture was measured using a standard compression test to a peeled, squared cut and cooked shrimp with a texture analyzer (TA-XT Stable Micro Systems, UK). A flat ended cylinder of 20 mm diameter was selected to mimic a human finger for compressing the samples. The samples were compressed to 30% of height of original sample height. The fleshy part of shrimp was cut into a square with dimensions of 8x8 mm^2 in order to maintain uniformity in size (Sigurgisladottir *et al*., 1999; Thybo *et al*., 1999). The average height of samples was 10 mm.

In order to construct the texture profile, the Texture Analysis (TA) software was programmed to apply double compression. The flat-ended cylinder (P35) approached the sample at the speed of 0.5 mm/s and penetrated 2 mm into the shrimp flesh with a holding time of 5 seconds. Then a second compression was made on the sample, force-

deformation curves were analyzed to calculate texture parameters (Sigurgisladottir *et al*., 1999; Thybo *et al*., 1999). Two basic textural parameters such as hardness (g) – maximum force of the first peak – and elasticity (s) were obtained. Other parameters of interest, such as cohesiveness (g.s), gumminess, and chewiness, were derived from the force vs. time plot. Using texture expert system software (developed by Stable micro systems Inc. UK) a double compression was performed on the same sample with a 1 second delay in between for comparison and calculation of parameters.

pH measurement

Samples were prepared by adding 20 ml of distilled water into 5 gm finely minced shrimp meat. The mixture was then homogenized by the help of mechanical homogenizer. pH of a representative homogenized sample was measured by a pH meter (EcoScan pH 5, EUTECH Instrument), which had been calibrated with standard pH buffers of 4 and 7 (AOAC, 1990).

Microbiological analysis

Conventional plate count method suggested by Maturin *et al*. (1998) was used to obtain total plate count from shrimp. Twenty-five grams of shrimp was mixed with 225 ml of sterile phosphate buffer solution (PBS) in a Stomacher bag and blended for 1 min. Three dilutions (10^{-1}, 10^{-2}, 10^{-3}) were prepared and 1 ml of each dilutions was inoculated in a sterile Petri dish. The solidified agar the Petri dishes were inverted and incubated for 48 ± 2 h at 35°C. Total viable counts were determined in terms of colony forming units (CFU) between 25-250, using a colony counter equipped with a magnifying glass.

TVB-N measurement

According to Lucke and Geidel's micro distillation method (Pearson, 1973), TVB-N measurements were made. The method includes steam distillation with MgO using the distillation apparatus. The principle of the method is that total volatile nitrogen is released by boiling flesh directly with MgO, which is then absorbed in boric acid. Amount of released volatile nitrogen is calculated by titrating with sulphuric acid. A shrimp sample weighing 5 g was macerated with 50 ml distilled water. MgO (about 1-2 g) was added, mixed well and then it was transferred into the distilling tube of a Tecator Distillation Apparatus (Model Kjeltec 1026). The distillation was carried out for 3 minutes and the distillate was titrated against 0.1N sulphuric acid. TVB-N was calculated in two replicates for each sample.

The value of TVB-N was calculated by Eq. 2 (Botta, 1995):

$$TVB-N\left(\frac{mgN}{100g\ felsh}\right)=\frac{Normality\ of\ acid\ x\ 1.4x100}{sample\ mass\ in\ g} \quad \text{Eq 2}$$

Sensory analysis

Trained panelists were requested to evaluate samples of cooked shrimp on a 3-point scoring scale (where 3 and 1 represented highest and least quality, respectively) for descriptive sensory parameters such as likeness vs. dislikeness of odor, appearance, and texture; SI was determined by Eq. 3 (Huss, 1995; Kreyenschmidt, 2003):

$$Sensory\ Index=\frac{2C+2O+1T}{5} \quad \text{Eq 3}$$

Where C = color, O = odour, T = texture.

Sensorial score was described against time by linear regression. Samples were considered "spoiled" when the SI reached 1.8 (Kreyenschmidt, 2003).

Data analysis

One way Analysis of Variance (ANOVA) was used to study the effect of storage temperature over time. Multiple linear regressions were used to measure response of time and temperature combination as independent variables against dependent variables of quality loss factors.

Temperature dependence of the quality loss rate is mathematically represented by the Arrhenius equation: Changes in intrinsic quality parameters (response variable y) were modeled with first order kinetic equation (Eq. 4).

$$y = e^{-kt} \quad \text{Eq 4}$$

The initial conditions: y =1 at t = 0 where k is rate of reaction, a constant (min^{-1}), and largely depends on temperature, therefore, a relationship with temperature is given by Eq. 5:

$$k=k_0\ \exp\left(\frac{E_a}{RT}\right) \quad \text{Eq 5}$$

Where k_0 is constant pre-exponential factor dependent on a range of temperature in reactions, E_a is the flow activation energy (KJ/mol ^oK), R is universal gas constant (8.314 kJ/mol.^oK), and T is absolute temperature (^oK).

Table 1. Total Change in Color at the end of storage period (84 hours)

Temp. °C	0	3	5	8
L-L_0	-4.65±0.11^a	-5.34±0.13^a	-7.03±0.04^b	-7.9±0.12^b
a-a_0	1.58±0.1^a	1.52±0.04^a	1.63±0.01^a	2.12±0.07^{ab}
b-b_0	-26.39±0.01^a	-25.58±0.2^a	1.63±0.01^b	1.25±0.01^b
ΔE	5.78±0.073^a	6.38±0.13^{ab}	8.01±0.02^{bc}	10.06±0.2^d

*Significantly different groups at P<0.05 are shown in superscript letters
**Calculated using Eq. 1.
***Measured in triplicate, ± = standard deviation.

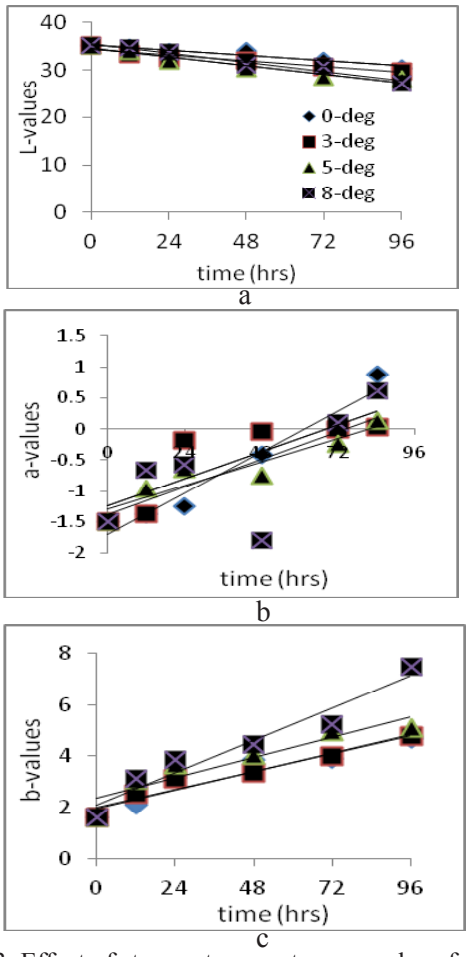


Figure 3. Effect of storage temperature on color of shrimp stored at 0-8°C: (a) Lightness (b) redness (c) yellowness

Results and Discussion

Effect of fixed storage temperature on quality parameters

Color degradation

The initial shining color of shrimps starts to look dull as soon as their death occurs. However, samples did not show much yellowness in the first 12 hours of storage at all temperatures. At temperatures 5 and 8°C, the samples appeared darker and blackening occurred near the upper region of a shrimp. Hard shell was removed before taking measurements. The color of underneath flesh changed from translucent white to yellowish-orange and finally to a dark color over time.

Table 2. Parameters of zero order regression of shrimp color at 0-8°C storage temperature

	Parameters	a-value	b-value	L-value
0°C	Slope	0.0253	0.03	-0.047
	Intercept	-1.66	1.98	35.2
	R^2	0.98	0.9	0.93
3°C	Slope	0.016	0.032	-0.075
	Intercept	-1.179	1.73	34.4
	R^2	0.71	0.99	0.95
5°C	Slope	0.0145	0.042	-0.076
	Intercept	-1.27	1.98	34.4
	R^2	0.89	0.98	0.95
8°C	Slope	0.0174	0.068	-0.08
	Intercept	-1.36	1.99	35.4
	R^2	0.5	0.98	0.95

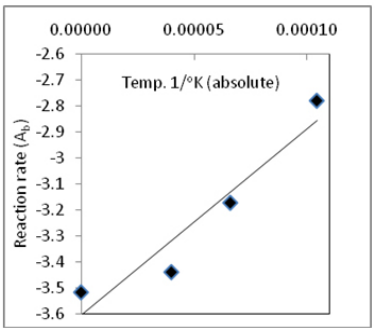


Figure 4. Relationship between color loss reaction rate and storage temperature (1/°K absolute)

Table 1 shows total change in color in terms of difference between final value and initial obtained from a color meter during the entire storage period. It can be seen that a temperature difference of 2-3°C had a significant effect on change in color; for example, b-value was significantly different at 3 and 5°C, whereas total change in color was significantly different at all storage temperatures.

Changes in L, a, b values of shrimp during storage could be conveniently modeled by a zero order equation (Eqs. 6-8):

$$L^* = A_L.t + L_0$$
$$a^* = A_a.t + a_0 \qquad \text{Eq 6-8}$$
$$b^* = A_b.t - b_0$$

where A_L, A_a, A_b are rate constants of the reaction, and t is the storage time (hrs). The parameters are summarized in Table 2 and Figures 3 (a, b and c).

Shrimp samples darkened with temperature and time; the lightness value at the end of storage period relative to the lightness at the day of harvest reduce linearly ($P < 0.05$) with time at each storage temperature (Figure 3a). The a-values (redness) increased over time and temperature without any visual appearance of red, which is an indication of cooking. The b values increased sharply over time

Table 3. Kinetic parameters of changes in textural quality of shrimps stored at 0-8°C

Texture attribute	E_a (KJ.Mole^{-1}.°K)	Reaction constant, k_0	Expression	R^2
Hardness (g)	-10.03	-0.0361	$\ln(H/H_0)=[46.93\ (1/T_{abs})-0.211]*t$	0.975
Elasticity (s)	-10.02	-0.0342	$\ln(E/E_0)=[405.53\ (1/T_{abs})-1.517]*t$	0.932
Cohesiveness (g.sec.)	-14.96	-0.0082	$\ln(C/C_0)=[23.634(1/T_{abs})-0.0946]*t$	0.899
Gumminess	-31.15	-0.0184	$\ln(G/G_0)=[(105.89(1/T_{abs})-0.405]*t$	0.961
Chewiness	-14.57	-0.0329	$\ln(Ch/Ch_0)=[119.79\ (1/T_{abs})-0.472]*t$	0.902

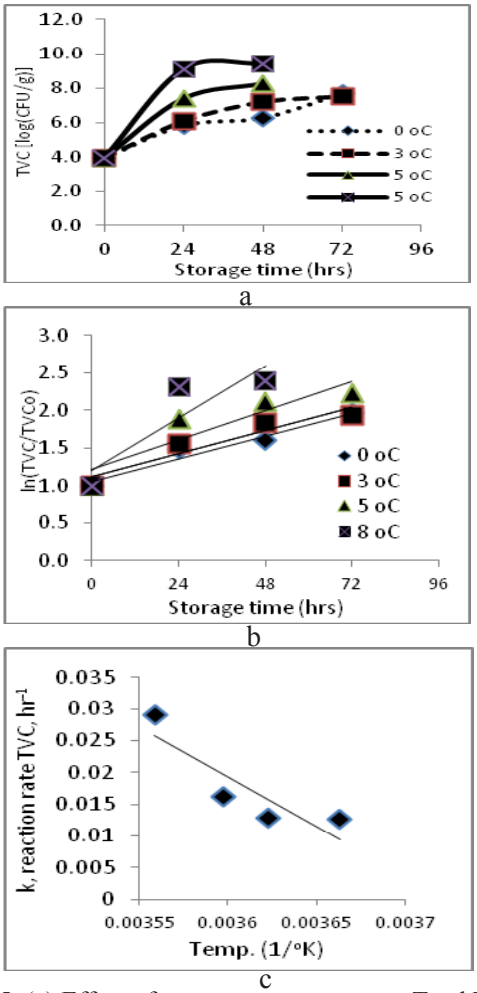


Figure 5. (a) Effect of storage temperature on Total Viable Count (TVC), (b) linear representation of normalized TVC (c) effect of temperature on the reaction rate, *k*.

at all temperatures, and fitted very well into a zero-order kinetic model. In all cases, a high correlation coefficient (R^2), ranging between 0.71 and 0.99, was obtained. However, at higher storage temperature of 8°C, a poor correlation was obtained due to onset of spoilage after 36 hours. Despite a significant variation in color in terms of Hunter parameters, a clear shift from yellow to red hues was not evident at a lower temperature range.

In order to determine temperature dependency of the reaction rate of yellowness, the A_b values obtained during linear regression (Figure 4) were plotted against absolute temperature (°K, 1/T+273). The resulting equation ($A_b = 7203.7(1/T_{abs}) - 3.6063$, $R^2 = 0.9105$) was inserted in Eq. 9.

$$b^*\text{-value} = [7203.7\ (1/T_{abs}) - 3.6063] \times t - 1.98$$

where the intercept (1.98) was fixed to initial b^*-value at the start of storage period. The activation energy (E_a) of the reaction was calculated as 60 (KJ. Mole^{-1}.°K) for a temperature range of 0-8°C, which signifies shrimp sensitivity to temperature. For quality monitoring purpose, b values can sufficiently be used to determine level of quality.

Changes in textural quality

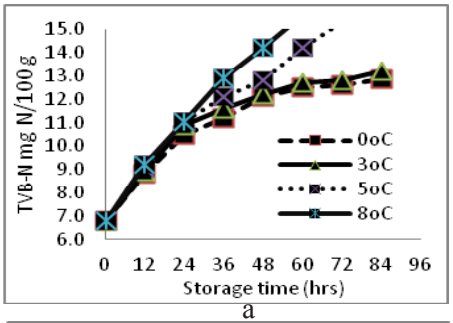
The initial values of texture attributes hardness (g), springiness (elasticity), cohesiveness (g.s), gumminess, and chewiness at zero storage time were: 115.02 ±1.5, 1.8 ± 0.034, 0.95 ± 0.01, 1.3 ± 0.026, 149.87 ± 2.5, and 142.38 ± 1.67, respectively. For simplicity, data obtained from texture analyzer of each attribute was normalized by dividing with the initial value and then plotted against storage time in a semi-log fashion to remove non-linearity, and then fitted with a first order equation yielding $R^2 > 0.9$ (Table 3).

Hardness and elasticity were observed decreasing significantly after 12 hours of storage. The samples appeared soft and mushy after 48 hours of storage at higher temperatures (5-8°C). Other attributes also showed the effect of deterioration, however, the rate of change was less than the hardness and elasticity, as indicated by the negative activation energy and the rates of reaction. Monaco *et al.* (2007) also reported high accuracy of prediction in the cases of hardness and springiness, as compared to the other TPA parameters. Although cohesiveness, gumminess, and chewiness are good indices for characterizing sensorial properties, their representation in accurate prediction was inadequate. Therefore, only hardness and elasticity were sufficient to model deterioration.

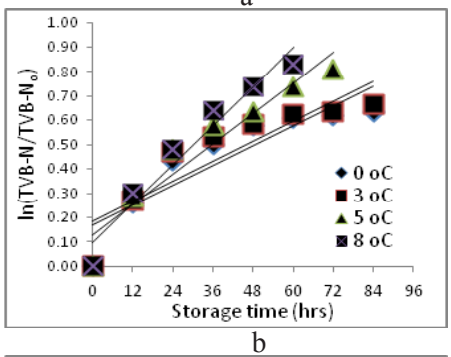
Changes in pH and microbial growth pattern

Initial pH of samples was 6.62, which steadily increased to 7.6. The results were in accordance with the published data (Riaz and Qadri, 1990) and also confirmed by the microbial growth, due to which bio-chemical changes took place. Bacterial contamination was estimated in terms of total viable cells: mesophilic and psychrophilic cell counts.

In general, TVC increased continuously throughout the storage period of 84 hours ($P < 0.05$) at all temperatures. The initial microbial load, in terms of viable cells, was 3.9 x 10^5 log (CFU/gm), which increased depending on the temperature (Figure 5). The initial load was higher, but within the acceptable industrial standard [<1 x10^6 log (CFU/gm)] and



a



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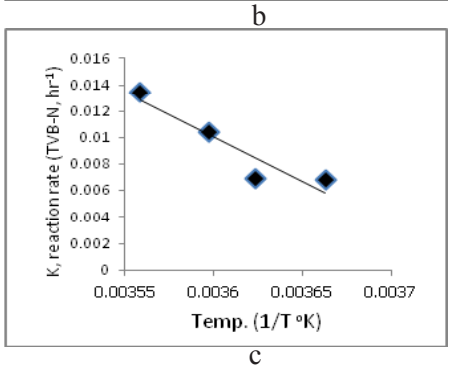


c

Figure 6. (a) Effect of storage temperature on total volatile basic nitrogen content in fresh shrimps (b) linear representation of normalized TVB-N (c) effect of temperature on the reaction rate, *k*.

values reported in the literature (Zeng, 2005). The samples were considered spoiled when total bacterial count reached 7.5 x 10^8 CFU/g. TVC was modeled (eq. 10) as shown in Fig. 5, yielding a high correlation coefficient ($R^2 > 0.9$):

$$TVC = e^{-(-155.8(1/T)+0.5802)t} \qquad \text{Eq 10}$$

(R^2 = 0.9-0.95)

Where:
TVC = total bacterial count [log(CFU/g)]
t = time (hours)

Changes in total volatile basic nitrogen

It was obvious that TVB-N increased as the storage time increased at all the storage temperatures. At at higher temperatures the change was steady, whereas at 0 and 3°C, the level of TVB-N tended to stabilize after initial increase. There was a significant difference in TVB-N levels after 24 hours of storage at all temperatures ($P < 0.05$). In some studies it has been reported that TVB-N decreased after an initial period of 4 days of storage (Ozogul *et al*., 2011). The possible explanation of such phenomena can be related to the experiments that have been conducted in iced storage, in which melting of ice washed away TVB-N, which results in decreased levels. In the current study, storage temperatures were maintained in a dry chamber; therefore, a decrease of TVB-N levels was not observed.

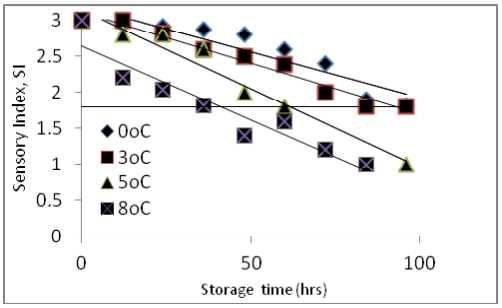


Figure 7. Change in Sensory Index during storage

It is recommended to use TVB-N if sensory evaluation indicates doubt about freshness. Depending on shrimp culture conditions and harvesting practices, a critical limit of 25-35 mg-TVB-N/100 g has been established (Einarsson, 2001).

A relatively poor correlation between storage time and TVB-N was observed at low temperatures (0-3°C, $R^2 = 0.7$), whereas the correlation improved at higher temperatures (5-8°C, $R^2 = 0.9$-0.94) when a linear curve was fitted (Fig. 6). The relationship between TVB-N, time and temperature of storage can be explained in exponential form (Eq. 11):

$$TVB-N = e^{(-67.279(\frac{1}{T_{abs}})+0.2523)t} \qquad \text{Eq 11}$$

(R^2 = 0.9-0.94)

Where:
TVB-N = total volatile basic nitrogen, mg N/100 g
T_{abs} = temperature (absolute), °K
t = time, days

Sensory evaluation

The sensory index (SI) decreased linearly with time at all storage temperatures. The samples were considered spoiled when SI reached 1.8 and below (Kreyenschmidt, 2003). As estimated, at higher storage temperatures, the spoilage point was reached faster than at the lower temperature. At 0°C, the samples were still in acceptable condition by the panelists by the end of the storage period. At higher temperatures and longer storage time, fruity, rotten, sulphydryl odors due to number of volatile aldehydes, ketones, esters, and sulphides produced by *Pseudomonas* spp. in iced seafood was noticed (Huss, 1995).

Table 4. Correlation between intrinsic quality parameters of shrimp stored at 0°C and sensory index

	L	a	b	Hardness, N	pH	TVC	TVB-N
a value	-0.955						
b value	-0.881	0.915					
Hardness, N	0.804	-0.827	-0.918				
pH	-0.769	0.745	0.829	-0.948			
TVC	-0.958	0.899	0.989	-0.972	0.992		
TVB-N	-0.787	0.774	0.913	-0.959	0.888	0.914	
Sensory Index	0.975	-0.959	-0.871	0.779	-0.828	-0.944	-0.635

($P < 0.05$)

Table 5. Results of step-wise multiple linear regression to estimate quality parameters of shrimps stored at variable storage conditions

Temp.	Predictors	a_0	a_1	a_2	a_3	R^2	SEE	F*
Hardness (N)	t	92.265	-1.154			0.896	12.48	51.667
	T_{bm}	120.442	-1.347	-6.972		0.965	7.96	68.313
	T_{bm} , t^2	127.877	-2.134	-6.431	.010	0.91	0.94	3.33E3
TBC	t	106.045	-1.380			0.887	15.63	47.140
	T_{bm}	124.758	-2.940	.019		0.980	7.28	119.729
	T_{bm} , t^2	139.755	-2.980	.018	-3.912	0.994	4.28	234.564
TVB-N	t	104.708	-1.390			0.881	14.89	52.656
	t^2	121.085	-2.75	.016		0.956	9.08	76.439
	t^2 , T^2_{bm}	133.502	-2.87	.016	-0.648	0.997	2.27	836.703
Sensory Index	t^2	3.04	-0.982			0.95	0.045	136.56

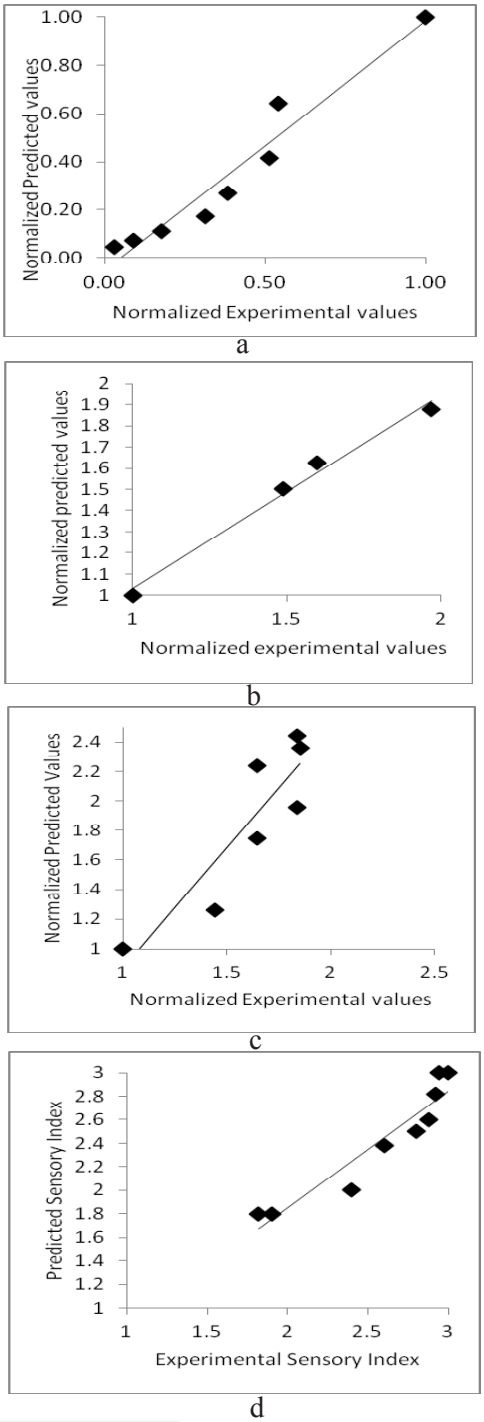
*T_{bm} = bulk mean temperature
**Significant $P < 0.05$

The correlation (R^2) between all storage temperatures and SI was between 0.85-0.9, which signifies that shelf life can be evaluated through subjective assessment using a 3-point sensory index (Fig. 7). The panelists were able to differentiate effect of constant storage temperatures on sensorial properties ($P < 0.05$) such as color, odour, and texture.

Inter-relationship between intrinsic quality parameters

A high correlation of 0.99 between TVC and pH was observed. Similarly, SI had a high and significant correlation between color values (L^*, a^* & b^*) and TVC. Low but significant correlation was observed with TVB-N and hardness.

A correlation matrix showed good relationship with high magnitude for most of the parameters (Table 4), confirming their significance to be used for shelf life prediction. It was also concluded that SI scores were in agreement with the instrumental methods.



	Slope	R^2
Hardness	1.0384	0.942
TBC	0.916	0.986
TVB-N	1.752	0.762
SI	0.892	0.996

Figure 8. Cross validation of developed models for (A) Hardness (N) (B) Total Viable Count [log (CFU/gm) (C) TVB-N mg/100 g (D) Sensory Index

Effect of variable storage temperature on quality parameters

In order to evaluate a collective effect of

Table 6. Determination of remaining shelf life and quality level at each distribution stage

Parameter	Loss in Quality Level at Different Distribution Stages*				Threshold quality level**
	First Stage (T_{eff}= 2°C)	2nd Stage (T_{eff}= 6.5°C)	Third Stage (T_{eff}= 3.5°C)	Fourth Stage (T_{eff}= 2°C)	
Hardness (N)	115	62	59	45	≤20
TVC log(CFU/gm)	3.9	5.8	6.2	7.7	≤7.5
TVB-N(mg/100 gm)	6.8	9.8	11.2	12.5	≤25
Sensory Index	3.0	2.94	2.92	2.8	≥1.8
Remaining Shelf life (hrs)	84	72	60	12	

*quality level at corresponding effective storage temperature (T_{eff}, °C), **as reported for TVB-N (Einarsson, 2001), TBC (Maturin, 1998), & Sensory Index (Huss, 1995) respectively. For hardness, the corresponding S.I. level was used.

different time and temperature scenarios, the bulk mean temperature (T_{bm}) function, as shown in Eq. 12 was used.

$$T_{bm} = \frac{\int T.dt}{dt} \quad \text{Eq 12}$$

T_{bm} is a constant temperature which induced a change in the product properties equivalent to the fluctuating temperature history during the same storage period (Solomon and Jindal, 2007) described by the sum of all time-temperature combinations averaged over entire storage period. The changes in quality parameters during storage under the fluctuating temperature conditions can be described by using stepwise multiple regressions in which the dependent variables were the normalized values (ratio of values at time t to initial value) of the quality parameter. The functional form of important parameters is as follows:

Quality Parameter	*Measured as*	*Functional Form*
Texture measurement	Hardness (N)	$\frac{H_t}{H_o} = f(T_{bm}, t)$
Total Viable Count	Log (CFU/gm)	$\frac{TVC_t}{TVC_o} = f(T_{bm}, t)$
Total Volatile Basic Nitrogen	TVB-N mg N/100g	$\frac{TVBN_t}{TVBN_o} = f(T_{bm}, t)$
Sensory Index	On a scale of 1-3	$\frac{SI_t}{SI_o} = f(T_{bm}, t)$

F-test significance determined which term to be included on the right hand side of the above equations. Table 5 shows predictors and corresponding coefficients of each equation. Four parameters of interest such as hardness, TVC, TVB-N, and SI were selected to measure their response to these time-temperature combinations. All combinations of predictors were significant at $P < 0.05$ from 0 to 3rd order polynomial arrangement (R^2 = 0.881-0.99, SEE = 0.045-15.63), except the SI for which only the square of time (t^2) was sufficient to predict sensory scores. This could be explained by the fact that human receptors cannot perceive the change in sensory quality accurately, which resulted in less variability in data, hence, yielding an equation with fewer terms.

Cross validation of developed models

By using another set of data that was not used for developing models, cross validation was performed. The values obtained from developed models were plotted against experimental values. The models were evaluated based on linear fit of data in terms of correlation coefficient (R^2) and the slope of the fitted line (Figure 8).

According to temperature profile depicted in Figure 2, four distinctive distribution stages were identified. By inserting average temperature at each stage with the models shown in Table 5, a quality level at each stage was determined. This allowed the estimation of remaining shelf life by using a pre-determined threshold level of quality. As it can be seen in Table 6, hardness and TVB-N did not reach the limit during 84 hours of storage, however, samples were unacceptable due to microbial growth.

Conclusion

The developed relationships between time-temperature history and quality parameters characterize the spoilage behavior of shrimps distributed through a cold chain and can be employed for predicting remaining shelf life of shrimps if time-temperature data is monitored and recorded at all stages of a supply chain. It was observed that a narrow temperature fluctuation of 2-3°C can significantly reduce the keeping quality and affect freshness. Therefore, measurement of the intrinsic quality indices by instrumental methods and human judgment, correlated with time-temperature history, is important to determine the quality level and remaining shelf life. Moreover, the developed kinetic models for representing the physical quality indices, such as textural and color properties in iso-thermal conditions with the kinetic parameters (reaction rate, activation energy), are useful for the development of stock replenishment schemes during distribution. Also, as in this study, a significant positive correlation was found between physical and chemical quality indicators, suggesting that simple measurement of pH and color parameters (L^*,b^*) can be used to predict TVC, TVB-N and hardness of shrimps, which are complex and difficult to assess at distribution centers and retail outlets. The empirical modeling approach, using bulk mean temperature function to account for the quality changes during fluctuating time-temperature regimes, provides an indication of quality level of samples. This information is important for pricing, grading, and shipment prioritization.

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Research Note

An automatic procedure for rapid online estimation of raw milk quality

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Received 1 September 2004; received in revised form 10 February 2005; accepted 15 February 2005

Abstract

A simple and inexpensive automatic procedure for rapid online assessment of raw milk microbiological quality was developed as an alternative to the presently available methods, which are either time consuming or require costly instrumentation. The technique was based on the monitoring of change in color of milk in methylene blue dye reduction test due to metabolic activity of the viable bacterial cells. The change in dye color was related to the output voltage signal of a specially designed light sensing probe (LSP) for about 40 min. The models developed for estimating SPC and MBRT from LSP output voltage showed R^2 of about 0.814 and 0.803, respectively. Subsequently, the output voltage of LSP was interfaced with a PC to predict and display the SPC (cfu/ml) and MBRT (h) of raw milk along with its quality grade in real time. The predictions of raw milk quality grades based on the calibration of LSP output voltage with SPC and MBRT were in good agreement with actual grades showing considerable promise for this novel assessment technique.

Keywords: Raw milk grading; Rapid quality detection; Methylene blue dye reduction; Light sensing probe

1. Introduction

Estimation of microbial load in raw milk is crucial in relation to its spoilage and keeping quality. Several techniques are currently available for determining the total viable cell count as well as microbial load. The laboratory methods for determining total viable cell count include direct microscopic count (DMC), most probable numbers (MPN), and standard plate count (SPC). However, the most frequently used methods for indirect estimation of the microbial load in raw milk in the dairies and milk collection centers are based on dye reduction. Among dye reduction methods, methylene blue reduction time (MBRT) is widely used at the milk collection centers all over Thailand for assessing raw milk quality grade.

The SPC requires sophisticated laboratory and expert personnel to perform the tests, and thus cannot be used for frequent routine analysis of milk quality for grading purposes. Several alternative methods have been developed in the past for determining the total viable cell count and relating to the SPC for rapid analysis (Fung, 2002). However, these methods require careful sample preparation, filtration, expensive dyes and reagents, and instrumentation. The Bactoscan measures the total bacterial count in raw milk by density centrifugation and continuous flow epifluorescent microscopy (Cunningham & Saunders, 1988). Shelef and Eden (1996) reported the use of a commercial instrument, BioSys™, manufactured by MicroSys, Inc. which detects metabolic changes in microorganisms during incubation using a newly developed optical methodology. Frundzhyan, Brovko, Babunova, Kartashova, and Ugarova (1999) optimized a rapid (30–35 min) bioluminescence assay for determining total bacterial

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0023-6438/$30.00
doi:10.1016/j.lwt.2005.02.010

contamination (TBC) of raw milk. Application of direct epiflourescent filter technique (DEFT) requires separation of bacteria from milk sample through centrifugation and then staining by acridine orange. Cady, Hardy, Martins, Dufour, and Kraeger, (1978) used an automated impedance measuring instrument to estimate microbial population in raw milk. These methods, of course, are capable of being used as an alternate to conventional methods in view of their high correlation coefficient ($r > 0.8$) with SPC. However, the associated equipments are quite expensive and sophisticated for general use.

There is a need for a simple, inexpensive and reliable method for rapid assessment of microbiological quality of raw milk at the farm level and milk collection centers. The MBRT estimates the bacterial load in milk indirectly and permits faster grouping of raw milk samples into different grades. The dye reduction time appears to be inversely related to the initial bacterial content of the sample (APHA, 1978). In general, the MBRT and SPC of raw milk samples have been reported to be in good agreement (Chuaprasert, 1984; Homhual, 2000).

Homhual (2000) proposed a technique for estimating the quality of raw milk based on measuring the change in blue color of dye-added milk over time by a light sensor. However, there were considerable variations in the output voltage patterns possibly due to the design of the electrical circuit and lack of temperature control mechanism. Therefore, the objective of present study was to further investigate the application of light sensing probes (LSPs) for assessing microbiological quality of raw milk in an independent manner. In addition, a secondary objective was to develop a computer-based automated procedure for online grading of raw milk in less than 1 h.

2. Materials and methods

Non-refrigerated raw milk samples ($n = 60$) were procured from the milk collection centers at three different locations in central Thailand, namely, Ayuthaya, Saraburi and Bangkok. Each milk sample was divided into three parts. One part was analysed using LSP, while the remaining two parts were incubated immediately for methylene blue test and SPC. Experiments were conducted on fresh milk samples collected over a period of 3 months (March–May, 2001).

2.1. Standard plate count

The total number of viable bacterial cells present in the raw milk sample was estimated by SPC method described in APHA (1978). The raw milk samples were subjected to serial dilution in 10^3 to 10^6 range and introduced to pre-sterilized nutrient media and agar in Petri-plates. The inoculated plates were incubated at $32 \pm 1\,^{\circ}C$ for 48 h. The total number of colony forming units (cfu) were counted and expressed in logarithmic form for simplicity.

2.2. Methylene blue reduction test

Methylene blue dye was prepared according to the method given in AOAC (1999). The dye solution and milk sample were used in 1:10 proportion on ml basis. The methylene blue reduction test was done according to the standard method (APHA, 1978). One milliliter of dye was transferred into test tube after placing 10 ml of sample. The test tubes were then incubated at $36 \pm 1\,^{\circ}C$ in hot water bath for 30 min and the change in color was carefully observed. In case the sample decolorized during the incubation period, the MBRT was recorded to be 30 min. After the initial 30 min reading, the subsequent readings were taken at hourly intervals.

2.3. Construction of light sensing probe

The probe was constructed using a cadmium sulfide-type light dependent resistor (LDR) to produce a voltage output signal proportional to the incident light. The LDR was enclosed in a thin glass tube which could be positioned in a test tube containing milk and dye mixture (1:10). The test tube was placed near the center of an opaque PVC pipe (10 cm dia and 40 cm long) to prevent the outside light. A 12 V halogen lamp was fixed at the bottom of the PVC pipe. The temperature inside the PVC chamber was controlled within $37 \pm 2\,^{\circ}C$ by a thermostat to turn an exhaust fan placed near the bottom of the pipe on and off (Fig. 1).

2.4. Data analysis

The output voltage from the LSP circuit was recorded at 1 min interval for a total duration of about 40 min. The voltage signals (V) at designated time intervals were normalized relative to their initial values (V_0) and used as independent variables. The MBRT and SPC of the raw milk samples were taken as dependent variables in the development of separate models by multiple linear regression analysis using SPSS ver. 10.0. The inclusion of independent variables in the model was based on F-test ($P \leqslant 0.5$).

3. Results and discussion

3.1. Calibration of light sensing probe

A typical plot of normalized output voltage from the LSP for raw milk samples of varying initial quality in

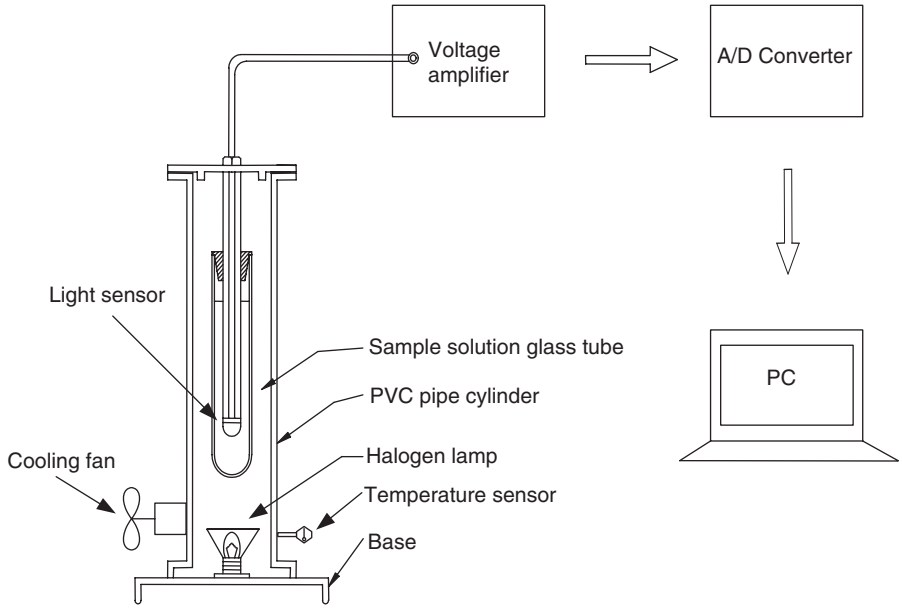

Fig. 1. Schematic diagram of experimental setup.

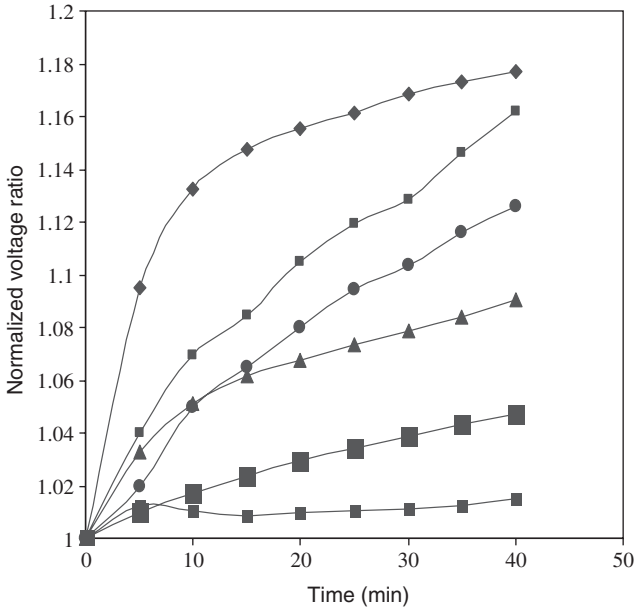

Fig. 2. Normalized voltage output of light sensing probe with time for raw milk samples indicating different methylene blue dye reduction times. MBRT (h): —◆— 0.5, —■—, —●— 2 hr,—▲— 3 hr, —■—, —■—.

terms of different MBRT is shown in Fig. 2. The probe output voltage increased over time with a decrease in the blue color of dye due to oxygen depletion by the bacteria present in raw milk sample. However, the whiteness of raw milk samples showed discernible differences initially. These differences in the appearance of the milk persisted even after the addition of methylene blue dye in raw milk causing the initial output voltage of the LSP to vary. Thus it was necessary to normalize the values of probe output voltage (V) recorded at an interval of 1 min relative to initial output voltage (V_0) at zero time. The normalized values of probe output (V/V_0) were then related to SPC and MBRT measurements separately using multiple regression in the following form:

$$\log(\text{cfu/ml}) = \sum a_i (V/V_0)_i, \quad (1)$$

$$\text{MBRT} = \sum b_i (V/V_0)_i, \quad (2)$$

where V_0 is the probe voltage output at start, V the probe voltage output at a given instant of time, a_i and b_i are regression coefficients, and i the subscript indicating test duration in minute (0,1,2,…,40).

The selection of independent variables in the regression models (Eqs. (1) and (2)) was based on F-test statistics ($P \leqslant 0.05$). The values of regression coefficients are given in Table 1. Statistical analysis showed R^2 and SEE for the estimation of SPC (log cfu/ml) to be about 0.814 and 0.526 and for MBRT (h) to be 0.803 and 2.358, respectively. These results indicated that monitoring the changes in the color of raw milk in methylene blue dye reduction test could be used for estimating microbial load in a relatively short period of time.

The models developed for estimating the microbial load in raw milk in terms of cfu/ml and MBRT were further validated using additional experimental data from 30 test samples. A comparison of predicted and experimental values of SPC and MBRT indicated fairly good agreement. The R^2 values were found to be about 0.852 and 0.806 for the calibration of SPC and MBRT, respectively, with slope of the regression line very close to 1 in both cases.

Table 1
Regression parameters of models developed for the calibration of light sensing probe

Standard plate count log(cfu/ml)		Methylene blue reduction time (h)	
Regression coefficients in Eq. (1)		Regression coefficients in Eq. (2)	
a_o	15.045	b_o	42.383
a_1	−16.269	b_1	−179.869
a_2	−4.652	b_2	84.834
a_3	33.909	b_3	3.148
a_4	−35.522	b_4	38.518
a_5	32.451	b_5	100.309
a_6	−44.377	b_6	−69.489
a_7	27.688	b_7	−39.086
a_8	−56.492	b_8	154.241
a_9	57.826	b_{10}	−205.569
a_{11}	69.042	b_{11}	119.547
a_{12}	-99.193	b_{13}	16.954
a_{14}	49.175	b_{14}	−133.867
a_{15}	−46.272	b_{16}	136.85
a_{19}	14.703	b_{19}	73.559
a_{21}	54.731	b_{21}	−15.068
a_{22}	−41.069	b_{24}	−120.743
a_{24}	−19.77	b_{26}	250.233
a_{26}	−9.77	b_{28}	−307.56
a_{32}	24.795	b_{32}	−82.786
a_{35}	52.476	b_{33}	−170.725
a_{38}	−36.927	b_{35}	166.78
a_{40}	−15.519	b_{38}	59.411
		b_{40}	80.125

3.2. Implementation of online scheme for assessing raw milk quality

The performance of LSP was analysed in terms of the differences in the output voltages of similar probes for a given milk sample. The output voltage when using multiple probes varied within a narrow range of 0.02–0.51 V. Similarly, the average differences between the estimated and experimental values of SPC (cfu/ml) and MBRT(h) for five raw milk samples were determined to be 2.48% and 0.42%, respectively.

In the next step, a scheme for online assessment of raw milk grade was implemented using a personal computer (PC) and LSP. The probe output voltage was amplified and passed through a data acquisition add-on A/D interface connected to the PC. A 32-bit Windows-based application (MilkoScan) was compiled in MS Visual Basic 6.0 to acquire the output signal of LSP in digital form at selected time intervals. The instantaneous values of probe output voltage were normalized for estimating the MBRT and SPC from the previously developed empirical models. The program could display the change in output voltage signal from the LSP in real time along with the predicted values of MBRT and SPC of the raw milk sample at the end of test duration as shown in Fig. 3. It was possible to customize the total duration of the test by users. Subsequently, the

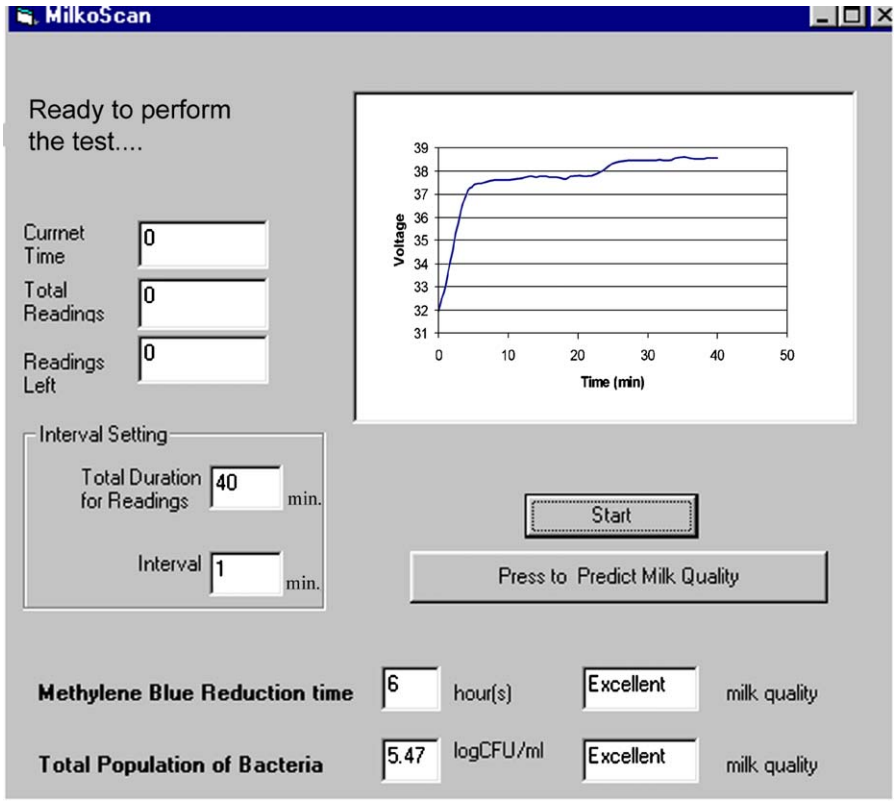

Fig. 3. Computer screen display of 'MilkoScan' for online assessment of raw milk quality.

Table 2
Scheme used for classifying raw milk samples into various quality grades

Grade of Raw milk	MBRT (h)	cfu/ml
Excellent	>5	$<2\times10^5$
Good	2–5	2×10^5–1×10^6
Fair	30 min–2	1×10^6–5×10^6
Bad	<30 min	$>5\times10^6$

Table 3
Classification of raw milk samples (%) into various quality grades based on MBRT, SPC and LSP ($n = 30$)

Quality grades	Classification based on			
	MBRT		SPC	
	Actual	LSP (predicted)	Actual	LSP (predicted)
Excellent	36.7	30	13.8	10.3
Good	33.3	40	41.4	41.4
Fair	30	30	31	31
Bad	0	0	13.8	17.2

model-predicted values of SPC and MBRT were used to classify the milk into arbitrarily selected excellent, good, fair and bad grades under tropical climatic conditions (Table 2).

A comparative evaluation of schemes used for classifying raw milk samples into various quality grades is presented in Table 3. It was obvious that the raw milk samples could be assigned quality grades based on MBRT or SPC according to the guidelines selected in this study. However, the application of SPC resulted in more stringent classification in comparison with MBRT. About 14% samples were assigned the lowest grade in case of SPC as compared to none based on MBRT. This was primarily due to the inherent errors associated with the estimation of the metabolic activity of the total cell population in milk samples by methylene blue reduction test. Interestingly, the predicted grades of raw milk by the LSP were in close agreement with actual classification based on the values of MBRT and SPC individually. Thus these results validated the application of LSP for monitoring the raw milk quality grades in a rapid and accurate manner.

4. Conclusions

It was shown that both SPC and MBRT could be used for classifying raw milk samples into various quality grades. However, the grading of raw milk based on MBRT appeared to be less stringent compared to SPC. A light sensing probe could be used for indirect estimation of SPC from the developed empirical model in test duration of about 40 min, and subsequently grade the raw milk samples in real time using an online PC-based system. This novel approach should prove to be of considerable practical significance for grading of raw milk inexpensively and rapidly especially at the milk collect centers.

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Agricultural Information Research 19(3), 2010. 64–70
Available online at www.jstage.jst.go.jp/

Original Paper

Prediction of Raw Milk Microbial Quality Using Data Mining Techniques

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Abstract

Data mining techniques were applied to predict raw milk quality in terms of methylene blue reduction time (MBRT) from the independent parameters of raw milk inspection parameters such as travel time, temperature of milk, solid-not-fat, %fat, acidity and specific gravity. Predictive models were developed and the performance of 3 data mining algorithms namely; Multiple Linear Regression (MLR), Artificial Neural Network (ANN) and K-Nearest neighbor (KNN), was measured in terms of average error and Root Mean Square Error (RMSE). MLR showed high and inconsistent RMS error in 3 randomly picked data partitions whereas KNN and ANN were able to predict the MBRT values from the physico-chemical quality parameters, KNN was the preferred algorithm (K=7, RMSE of 1.7). The models were applied to a new set of data (n=78) without showing them the output parameter (MBRT). The predicted values of MBRT were plotted against the actual observed values to classify milk into 4 quality grades.

Keywords

Raw milk, microbial quality, data mining, ANN, KNN, dairy

Introduction

Raw milk is subjected to various quality tests when arrived at processing plants such as %fat, solid not fat (SNF), %Acidity, specific gravity etc. These types of physical and chemical test are common and routinely conducted to classify the milk into quality grades for pricing purpose. In order to assess microbial quality of raw milk standard plate count (SPC) is recommended. SPC involves sampling of raw milk, inoculation into growth media and incubated at favorable temperature that would allow growth of bacteria. The number of bacteria is counted in terms of colony forming units per ml of milk (CFU/ml). SPC requires at least 48 hours to classify milk into quality grades such as if CFU/ml $<2\times10^5$ the milk sample will be graded as 'excellent', however, if the sample contain $>5\times10^6$ CFU/ml of milk the milk sample would be 'bad' (IDF 1986). Therefore, this is not practical test to perform on arrival of milk lot to appreciate the price, milk industry relies on an indirect method of quality assessment; methylene blue reduction time (MBRT). In this method a blue dye is added to milk which over time becomes white due to metabolic activity of bacteria. If more bacteria are present in the sample, faster the metabolic activity will be which result into disappearance of dye in the milk (APHA 1992). Several studies have proved a strong correlation (R^2 0.81–0.89) of MBRT method with SPC (Homhual 2000, Ahmad 2001). However, a good milk sample would take 8–10 hours to yield results which are still a relatively longer time from operational point of view.

There has always been a need to establish alternative methods to rapidly assess microbial quality which are robust and reliable yet simple and inexpensive. Some rapid methods include density centrifugation and continuous flow epifluorescent microscopy (Cunningham and Saunders, 1988), use of a commercial instrument, BioSys™, manufactured by MicroSys, Inc. which detects metabolic changes in microorganisms during incubation. Bioluminescence assay for determining total bacterial contamination (TBC) of raw milk (Frundzhyan et al. 1999), direct epiflourescent filter technique (DEFT) requires separation of bacteria from milk sample through centrifugation and then staining by acridine orange, impedance measuring instrument to estimate microbial

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population in raw milk (Cady et al. 1978). Attempts have been made to assess microbial quality by measuring dissolved oxygen (DO) in milk sample and its reduction over time. The idea was to relate the rate of change in (DO) levels with the population of bacteria in the sample (Homhual and Jindal 2001). MBRT method was modified into an automatic scheme using light sensing probe (Ahmad and Jindal 2006). This procedure was capable to producing the results in less than 1 hour.

Homhual (2000) related routine analysis of raw milk with microbial quality (MBRT) by using step wise multiple linear regression which yielded a poor correlation. The data was then subjected to artificial neural networks (ANN) to model the behavior. In training and validation models, a fairly good correlation was found. Ahmad and Jindal (2006) related output voltages obtained from a light sensing probe (LSP) with the MBRT and SPC using multiple linear regressions. It was reported that by improving the design of sensing probe, input power stabilization and temperature stabilization reduces inherent discrepancy in the data. A good correlation was obtained as (R^2) 0.89 and 0.91 between SPC and MBRT against LSP, respectively. The model was validated with new data and was reported as workable, however, with 30 or more coefficients (out of 40), the model was not convenient to use and implement. Attempts to further reduce the predictors were not successful.

Data mining techniques have been successfully implemented in predictive modeling of biological systems (Anpalaki 2008). However, its application penetration in agricultural and biological systems is rarely reported.

Multiple Linear Regressions (MLR)

MLR predicts the outcome of new cases and relates a quantitative outcome with predictors. By separating data into training and validation data sets and using the coefficients obtained in training the data to estimate new observations (predictions) in validation data. This arrangement can then be used for estimating the error in predictions without having to assume that the noise follows a normal distribution. Predicted values are compared with the values of the dependent variable that was actually observed in the validation data. This allows estimating the accuracy of the models by measuring average of sum of squares.

***k*-Nearest Neighbor (KNN)**

We chose *k*NN algorithm because it seems more appropriate with the data and also it does not make assumption about the form of the relationship between the input variables and predictors. The non parametric method, unlike MLR, does not involve estimation of parameters. It finds similarities between the predictors' values. The measure of distance is performed by Euclidean distance between the neighbors. The simplest case would be k=1 and this can be extended until error minimizes between observed

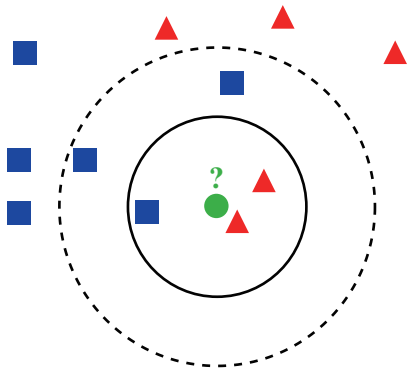

Fig. 1 An example of *k*NN algorithm (*courtesy: Wikipedia*)

(x_1, x_2, ..., x_p) and predicted (v_1, v_2, ..., v_p) values.

$$\sqrt{(x_1-\upsilon_1)^2+(x_2-\upsilon_2)^2+\ldots+(x_p-\upsilon_p)^2} \quad (1)$$

In the *k*NN classification, the test sample (circle) should be classified either to the first class of squares or to the second class of triangles. If k=3 it is classified to the second class because there are 2 triangles and only 1 square inside the inner circle (Fig. 1). If k=5 it is classified to first class (3 squares vs. 2 triangles inside the outer circle) (Cover and Hart 1967).

The purpose of this algorithm is to classify a new object based on attributes and training samples. The classifiers do not use any model to fit and only based on memory. Given a query point, we find k number of objects or (training points) closest to the query point. The classification is using majority vote among the classification of the K objects. Any ties can be broken at random. k Nearest neighbor algorithm used neighborhood classification as the prediction value of the new query instance (Shmueli et al. 2007).

Artificial Neural Networks (ANN)

The potential drawback of regression models of specifying type of relationship limits its use in complicated data sets. Biological processes are highly non-linear in nature and offers difficulty when developing predictive models using conventional statistical methods. ANN itself learns the form of relationship existed in data. Their ability to learn from a set of examples makes them a very flexible and powerful tool for modeling the complex systems. ANN offers a wide range of different architects that can be used depending on the complexity of the problem. In general, however, an ANN consists of an input function that computes the weighted sum of the input, activation function that transforms the weighted sum into final value.

$$in_i - g \cdot \left[\sum_j W_{ji} \cdot a_j\right] \quad (2)$$

Where

in_i=input function
g=activation function
W_{ji}=weight
a_j=activation value

In all the above discussed data mining techniques, data is divided into training and validation sets. This way prediction error can be compared in training and validation data. Prediction error can be simply measured by average error; however, it does not reflect the size of errors. Likewise, total sum of squared errors has the same draw back. Root mean square (RMS) error, on the other hand, allows us to compare if error is larger or smaller in the validation data when compared to the training data.

Objective of the study

It is hypothesized that microbial population brings biochemical changes in raw milk such as %fat, SNF, specific gravity. The number of microbes present in milk sample is directly influenced by travel time and temperature during travel and at arrival. Current paper evaluates data mining algorithms to develop a model that could fairly predict microbial classification of raw milk by routine analysis data.

Practical application

A robust and speedy model would provide basis for an automated scheme of raw milk price appreciation and quality evaluation at farm level as well as in processing plants. It is estimated quality can be evaluated in less than one hour without waiting for results from dye reduction method.

Materials and Methods

Data collected during 2006–2007 from a local dairy in the suburbs of Bangkok Thailand. Total 280 data pattern were used. Each data were obtained from samples drawn from the same milk lot. The parameters were namely: travel time, milk temperature on arrival, %fat (BSI 1959), SNF, SG, acidity and corresponding MBRT (h).

Detail of each testing method is given in their respective laboratory manual (APHA 1992).

Data analysis

In order to employ supervised learning algorithms, data set was divided into 2 groups. One of the groups was further subdivided into training data, model validation data and model testing data sets. 2nd group of dataset was kept aside and was not used in training or validating models. It was used to deploy model on this new data where the output was known for independent verification of the model.

We compared multiple linear regression (MLR), Artificial Neural Network (ANN) and *k*-nearest neighbor (KNN):

MLR: Independent variables SPC and MBRT were estimated from properties of raw milk arriving at dairy plant. Accuracy of predicted values was determined by Average error, total sum of squared errors and root mean squared error in (i) training dataset (ii) validation data set (iii) test data set. In addition, experimental and fitted values were plotted linearly to validate the model using R^2 and Standard Error of Estimate (SEE).

ANN: We used 3 layer feed forward neural network (NN) architecture and a nonlinear, sigmoid transfer function was employed. There was only one neuron (MBRT) in the input layer. The nodes in each layer accept the input values and successive layers of nodes receive inputs from previous layers (hidden layers). It was observed that one hidden layer was sufficient to approximate non linear function. Number of neurons in the hidden layers affects the output error function during the network training. Too few hidden neurons may limit the ability of the neural network to model the process, and too many can result in mimicking the noise present in the dataset (Shmueli et al. 2007). Other factors, such as learning rate, momentum term and noise were employed in the ranges of 0.1–2.0; 0.1–1.0 and 0.01–0.1 respectively. The optimum value was chosen when lowest training error was obtained. By using Random Centroid Optimization procedure provided by the software following optimum values were determined learning rate, 1.39; momentum term, 0.6 and noise, 0.01. There were 25 nodes in the hidden layer.

The last layer (output layer) contained 6 neurons namely; travel time, milk temperature on arrival, %fat, Solid-not-Fat, specific gravity and acidity.

Accuracy of prediction was determined by average error, total sum of squared errors and root mean squared error in all 3 data sets. In addition, experimental and fitted values were plotted linearly to validate the model using R^2 and Standard Error of Estimate (SEE).

kNN: The algorithm was applied in the similar fashion to all 3 data set and accuracy was determined as described for MLR. The critical aspect was to choose value of *k* or the number of neighbors. We initially run the algorithm for k=1 to 20. The optimum value was obtained where %error in training and validation sets was minimum.

XLMiner ver. 3.0 was used to carry out the predictive analysis (Shmueli et. al. 2007).

Results and Discussion

Travel time varied between 1 to 14.5 hours. In fact, an estimated travel time from 5 different suppliers location was established. However, in practice, the recorded time was the time between loading at suppliers location and unloading at the

processing plant including weighting time for quality testing etc. Therefore it varied greatly. Temperature of milk was recorded immediately after it is pumped from the tanker which ranged from 1 to 8°C. It was obvious that longer travel time and higher temperatures directly affect the microbial population in milk. There has been no established relationship, however, between acidity, SNF, %fat and specific gravity with the MBRT. Therefore, in this study, non-parametric approach (ANN, KNN) was taken whether pattern classification supports the hypothesis that changes in biochemical properties are due to the presence microbial population and that algorithms could classify the patterns correctly without, necessarily, understanding the underlying process.

Whole data set (n=200) was partitioned into training, validation and test data sets. The partitions were done by randomly drawing the values from the whole data set to avoid any accidental bias. Three random partitions were generated using different initial seed values of (12345, 2567, 1369) for randomization before running predictive algorithms of MLR, ANN and KNN. The data was trained at 50% of data patterns which was used to build models, the validation partition (of 30% data patterns) was used to see how well the model performed on new data set, and finally remaining 20% data patterns were used in test data set. Test data set was used to remove any doubt of 'bais' in data by comparing RMS error of test data with that of validation data. This practice is useful to assess how well final model might perform on new data.

All models were compared in terms of average error and RMS error in training (50%), validation (30%) and test (20%) data sets. Since average error does not reflect the size, type and polarity of error, therefore, RMSE was used for model selection.

MLR

Relationship was developed for estimating microbial quality of raw milk in terms of MBRT (h) from routine analysis tests by multiple linear regression. In training data set (n=100), MLR yielded low average error which indicated an overfitting of data into model which could be confirmed by high RMS error (7.4) in test data set (Table 1). In variance-covariance matrix of predictors, variable 'Specific Gravity' was also ignored due to multicolinerity. Multi-colinearity is the presence of two or more predictors sharing the same linear relationship with the output variable. It can be that dropping one or more correlated predictors reduces the variability in data and can increase the average error (or bias) of prediction. ANOVA analysis yielded a low adjusted *R-square*, low *p-value* and high residual sum of squares suggested MLR to be an unsuitable algorithm for predicting microbial quality from given data patterns. A low R^2 value was reported when MLR (stepwise) was used to predict MBRT from routine analysis (Homhual 2000) which conform the current finding. The potential weakness of this algorithm is that it is fragile, being time consuming and unstable, therefore, at each step we dropped the predictors which were not statistically significant. The algorithm stops when predictors left with significant contributions. Generalizations of MLR models may produce erroneous results especially in biological systems. Table 1 shows RMSE of 3 data sets and 3 random partitions which clearly indicate inconsistency in prediction error, therefore, MLR was not used in further predictive modeling.

ANN

The architecture of this algorithm was consisted of 1 hidden layer with 25 nodes and 30 epochs. Error tolerance was set at 0.01 while optimum weight of activation function was 0.6. Randomly drawn training (50%), validation (30%) and test (20%) datasets from the whole dataset show an initial high RMSE and with the number of epochs (number learning neurons passes) the RMSE minimized. No further decrease in RMSE was observed by increasing number of epochs of by introducing new weight function. In order to avoid overfitting, the algorithm was stopped at once lowest and 'reproducible' RMSE (of 2.3, 1.9, and 2.32) in test data set was reached (Table 1).

The primary issue in applying *k*-nearest neighbor algorithm is to find the optimum value of *k* which has the best classification performance. In order to avoid over fitting by choosing a low value of *k* or reducing the variability in data by selecting a higher value of *k* (as it tends to assign the records to fewer classes), we run algorithm with *k*=1 to 20. The *k* which has the

Table 1 Comparison of performance of algorithms in terms of RMSE

Algorithm	Dataset	Random set 1	Random set 2	Random set 3	Avg. RMSE
MLR	Training Data scoring	1.65	1.68	1.55	1.63
	Validation Data scoring	1.75	1.66	14.07	5.83
	Test Data scoring	7.44	5.73	1.92	5.03
ANN	Training Data scoring	2.03	2.08	1.71	1.94
	Validation Data scoring	2.11	2.07	2.05	2.07
	Test Data scoring	2.29	1.89	2.32	2.17
KNN	Training Data scoring	0.25	0.21	0.20	0.22
	Validation Data scoring	1.80	1.88	1.75	1.81
	Test Data scoring	1.71	1.67	1.81	1.73

Training data (n=100), Validation data (n=60), Test data (n=40)
Random set 1, 2 and 3 were obtained by changing random seed during data preparation *k*NN

Table 2 Validation error log for different *k*

Value of *k*	Training RMS Error	Validation RMS Error	
1	0.248394847	1.939444662	
2	0.248394847	1.909533577	
..	.....	.....	
6	0.248394847	1.802937268	
7	**0.248394847**	**1.796108408**	← Best *k*
8	0.248394847	1.800640901	
..	.....	.....	
18	0.248394847	1.812628183	
19	0.248394847	1.821036371	
20	0.248394847	1.825155905	

best classification performance in terms of computing errors in both training and validation sets, was selected. In our case, *k*=7 showed minimum of misclassification rate (error) in the validation set (Table 2).

Therefore, *k*-nearest neighbor algorithm was run with *k*=7 on training, validation and test datasets. Average error and RMSE of the selected model is given in Table 1. Given the low values of average error and RMSE as compared to ANN, *k*-NN could be selected for predicting microbial quality from the predictors that apparently has no relationship with the output variable. This is due to the nature of algorithms that does not make assumptions about the form of the relationship between output variable and predictors (like MLR). *k*-NN is basically a classification algorithm, non-parametric in nature that draws information from similarities between predictors' values of the dataset.

Performance of algorithms on new data set

In order to independently evaluate the performance of algorithms and to avoid any accidental bias in model generalization, an unseen set of data of predictors such as transport time, temperature, SNF, %fat, SG and acidity; were used to generate MBRT values. ANN and KNN models performed very well (Fig. 2) when the predicted values of MBRT were compared with observed MBRT values of corresponding milk samples (n=78). In terms of lowest RMSE in test set (KNN=1.73) and distribution of predicted values around the observed values, KNN was preferred over ANN model. According to the raw milk quality classification given in Appendix A, all algorithms were able to

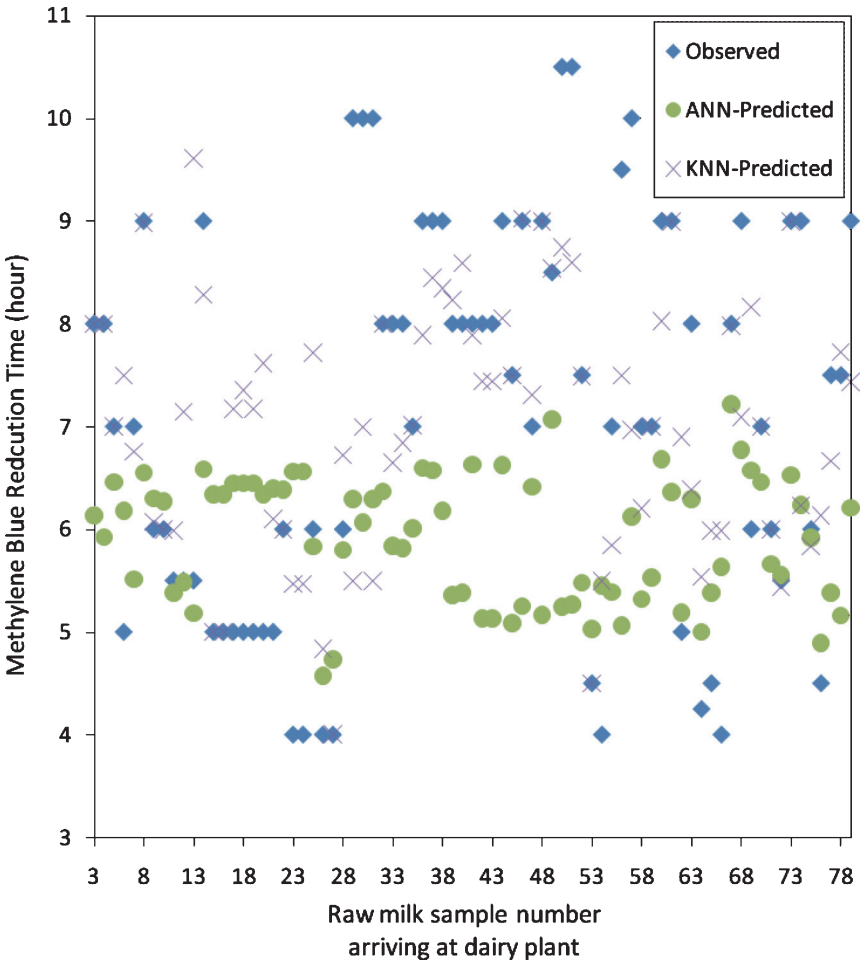

Fig. 2 Observed and predicted microbial quality of raw milk

correctly predict the MBRT values within the range of 3 grades i.e., excellent (>6 hours), good (4 to 6 hours) and fair (1 to 4 hours). Note there was no 'fair' and 'bad' samples since all data were taken from commercial operation. There was no misclassification as 'extreme cases' that a low quality grade had been assigned a higher grade.

Future work

Microbial quality assessment is very important factor in quality evaluation and safety of consumers. However, industry is striving to find practical solution to confidently estimate active microbial population. MBRT is also an indirect estimation technique and is used due to its easy handling by laboratory staff. SPC, on the other hand, is the standard technique which is conducted to confirm the microbial quality assessment tests. Strong relationships have been reported between MBRT and SPC, therefore, it should be possible to classify raw milk samples based on SPC which will give more weightage to the predictive models. Another future development could be the prediction of one or more types of bacterial instead of whole microbial population which is relatively easier to perform.

Conclusion

Data mining techniques can be successfully used in biological systems especially where relationships between predictors and the output variables are not well established or difficult to explain. Also, in situations where linear regression could not yield satisfactory results due to its tendency to ignore co-linear parameters, data mining approaches could be an alternative since they are dependent on full set of observation. If there is little or no observational history, data mining has little or no role in prediction. The critical factor in applying data mining techniques is to find the optimum training parameters in order to avoid over or under fitting which can yield misleading results.

Acknowledgements

This paper is based on the datasets obtained from a local dairy in Thailand. We have not included lengthy tables and graphs obtained during modeling work but can be provided upon request. XLMiner® is data mining add-in in Excel and can be downloaded from www.xlminer.com

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Received December 7, 2009
Accepted May 24, 2010
Agro-informatics & Technology

Appendix 1 Classification based on predictive values of MBRT from developed models using data mining algorithms (XLMiner®)

Sample	Observed		Predictive			
	Actual		ANN		KNN	
	MBRT (h)	Grade	MBRT (h)	Grade	MBRT (h)	Grade
1	6	excellent	7	excellent	9	excellent
2	6	excellent	6	excellent	8	excellent
3	8	excellent	6	excellent	8	excellent
4	8	excellent	6	excellent	8	excellent
5	7	excellent	6	excellent	7	excellent
6	5	good	6	excellent	8	excellent
7	7	excellent	6	good	7	excellent
8	9	excellent	7	excellent	9	excellent
9	6	excellent	6	excellent	6	excellent
10	6	excellent	6	excellent	6	excellent
11	5.5	good	5	good	6	excellent
12	5.5	good	5	good	7	excellent
13	5.5	good	5	good	10	excellent
14	9	excellent	7	excellent	8	excellent
15	5	good	6	excellent	5	good
16	5	good	6	excellent	5	good
17	5	good	6	excellent	7	excellent
18	5	good	6	excellent	7	excellent
19	5	good	6	excellent	7	excellent
20	5	good	6	excellent	8	excellent
21	5	good	6	excellent	6	excellent
22	6	excellent	6	excellent	6	excellent
23	4	good	7	excellent	5	good
24	4	good	7	excellent	5	good
25	6	excellent	6	good	8	excellent
26	4	good	5	good	5	good
27	4	good	5	good	4	good
28	6	excellent	6	good	7	excellent
29	10	excellent	6	excellent	6	good
30	10	excellent	6	excellent	7	excellent
31	10	excellent	6	excellent	6	good
32	8	excellent	6	excellent	8	excellent
33	8	excellent	6	good	7	excellent
34	8	excellent	6	good	7	excellent
35	7	excellent	6	excellent	7	excellent
36	9	excellent	7	excellent	8	excellent
37	9	excellent	7	excellent	8	excellent
38	9	excellent	6	excellent	8	excellent
39	8	excellent	5	good	8	excellent
40	8	excellent	5	good	9	excellent
41	8	excellent	7	excellent	8	excellent
42	8	excellent	5	good	7	excellent
43	8	excellent	5	good	7	excellent
44	9	excellent	7	excellent	8	excellent
45	7.5	excellent	5	good	8	excellent
46	9	excellent	5	good	9	excellent
47	7	excellent	6	excellent	7	excellent
48	9	excellent	5	good	9	excellent
49	8.5	excellent	7	excellent	9	excellent
50	10.5	excellent	5	good	9	excellent
51	10.5	excellent	5	good	9	excellent
52	7.5	excellent	5	good	7	excellent
53	4.5	good	5	good	5	good
54	4	good	5	good	6	good
55	7	excellent	5	good	6	good
56	9.5	excellent	5	good	8	excellent
57	10	excellent	6	excellent	7	excellent
58	7	excellent	5	good	6	excellent
59	7	excellent	6	good	7	excellent
60	9	excellent	7	excellent	8	excellent
61	9	excellent	6	excellent	9	excellent
62	5	good	5	good	7	excellent
63	8	excellent	6	excellent	6	excellent
64	4.25	good	5	good	6	good
65	4.5	good	5	good	6	excellent
66	4	good	6	good	6	excellent
67	8	excellent	7	excellent	8	excellent
68	9	excellent	7	excellent	7	excellent
69	6	excellent	7	excellent	8	excellent
70	7	excellent	6	excellent	7	excellent
71	6	excellent	6	good	6	excellent
72	5.5	good	6	good	5	good
73	9	excellent	7	excellent	9	excellent
74	9	excellent	6	excellent	6	excellent
75	6	excellent	6	excellent	6	good
76	4.5	good	5	good	6	excellent
77	7.5	excellent	5	good	7	excellent
78	7.5	excellent	5	good	8	excellent
79	9	excellent	6	excellent	7	excellent

Classification criterion: >6=excellent, Good=4–6, Fair=1–4, bad=<1 hours (Department of Dairy Promotion, Thailand)

Journal of Stored Products Research 54 (2013) 48–53

Contents lists available at SciVerse ScienceDirect

Journal of Stored Products Research

journal homepage: www.elsevier.com/locate/jspr

Efficacy of atmospheric and pressurized carbon dioxide or air against *Sitophilus zeamais* Motchulsky (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) in milled rice

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ARTICLE INFO

Article history:
Accepted 7 May 2013

Keywords:
Sitophilus zeamais
Tribolium castaneum
Milled rice
Carbon dioxide
High pressure

ABSTRACT

Carbon dioxide gas was evaluated in the laboratory for control of *Sitophilus zeamais* Motchulsky and *Tribolium castaneum* (Herbst) in milled rice using a specially designed pressure chamber. Tests were conducted at atmospheric pressure with 60, 80 and 100% carbon dioxide and with 100% carbon dioxide pressurized to 4, 6 and 8 bars. LT_{99} were ≤79, ≤78, and ≤148 h for all life stages of *S. zeamais* exposed to 60, 80 and 100% carbon dioxide, whereas, they were ≤59, ≤51 and ≤66 h for *T. castaneum*. Pure carbon dioxide controlled the adult stage, but there tended to be no significant differences of carbon dioxide concentrations on mortality of immature stages. When pure carbon dioxide was pressurized, mortalities of the two insect species were increased significantly. LT_{99} values decreased with levels of applied pressure: ≤29, ≤9.0 and ≤4.8 h for *S. zeamais* and ≤15, ≤5.8 and ≤2.3 h for *T. castaneum* at pressures of 4, 6 and 8 bars, respectively. *Sitophilus zeamais* was more tolerant than *T. castaneum* to carbon dioxide gas. The adult was the most susceptible stage, however, immature stages responded differently to carbon dioxide gas applied at varying concentrations and pressure levels. Adult mortalities were lower when air was used in the pressure chamber instead of carbon dioxide. Pressurized carbon dioxide at relatively low pressure (4–8 bars) was found effective in controlling all live stages of *S. zeamais* and *T. castaneum* in milled rice with shorter exposure times than at atmospheric pressure using an inexpensive set of equipment as compared to high pressure carbon dioxide fumigation (20–30 bars).

1. Introduction

One of the most important problems faced during storage of milled rice is insect infestation. The major species of stored product insects found in rice mills in Thailand are the Maize Weevil, *Sitophilus zeamais* Motchulsky, and the Red Flour Beetle, *Tribolium castaneum* (Herbst). Hidden infestations of insect eggs inside and outside rice kernels cause significant quality loss when insects hatch from eggs develop in the sealed plastic packages. As a common practice, rice is treated with chemical fumigants to prevent insect development. However, with increasing consumer demands for chemical-free food products, there have been a number of attempts to introduce other insect control methods to replace fumigation.

Carbon dioxide could be an alternative to chemical fumigants as it is safe to the environment but can kill insects. Most stored product insects are killed under atmospheres of <3% O_2 or >40% CO_2 (Bailey, 1965). To obtain a complete kill of all growth stages, carbon dioxide fumigation requires lengthy exposure periods. Annis (1987) reported that *Sitophilus oryzae* L. (rice weevil) was amongst the most tolerant stored product insects to carbon dioxide rich atmospheres. The proposed exposure time to control *S. oryzae* when using >40% carbon dioxide at 25 °C was 15 days, which was assumed to be adequate for all other species. However, to obtain high acute mortality for this kind of insect, high concentrations of carbon dioxide are needed but with nil oxygen effectiveness is reduced. Lindgren and Vincent (1970) found that atmospheres with 40–90% of carbon dioxide in air were more toxic to *S. oryzae* adults

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0022-474X/$ – see front matter © 2013 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jspr.2013.05.002

than 100% carbon dioxide. Similarly, Annis and Morton (1997) reported that atmospheres with 65–95% of carbon dioxide were more effective at killing all life stages of *S. oryzae* than pure carbon dioxide. Even at a carbon dioxide concentration as high as 95%, an exposure time of several days was required to kill all developmental stages of *S. oryzae* (Annis and Morton, 1997). Among the various species of weevils that attack stored grain, *S. oryzae* was reported to be more tolerant to carbon dioxide than the granary weevil, *Sitophilus granarius* (L.) (Lindgren and Vincent, 1970), but there is no published information on the susceptibly of the maize weevil *S. zeamais*. In the case of *T. castaneum*, many researchers have studied on the effect of pure carbon dioxide gas or carbon dioxide rich atmospheres on the mortality of adults (Press and Harein, 1967; Jay and Pearman, 1971) and also other life stages (Harein and Press, 1968; AliNiazee, 1971). Adults of *T. castaneum* are more tolerant than *S. oryzae* (AliNiazee, 1971) in atmospheres containing 45–80% of carbon dioxide, however shorter exposure times are required to kill all development stages of *T. castaneum* when using carbon dioxide 100% (Press and Harein, 1967; Annis and Morton, 1997). Each insect life stage responds differently to carbon dioxide. For almost stored product insects studied, pupae are the most tolerant stage whereas adults are the most susceptible stage, so any new studies should include non-adult stages.

The toxicity of carbon dioxide against stored grain insects has been reported by many researchers but most of these studies have been conducted at atmospheric pressure (such as the studies reviewed above) or reduced pressure (Locatelli and Daolio, 1993). Carbon dioxide fumigation at less than atmospheric pressure requires longer time than chemical fumigation such as methyl bromide or phosphine. To shorten exposure time, Nakakita and Kawashima (1994) introduced the effective use of carbon dioxide gas treatment under pressure at 5–30 bars followed by a sudden releasing of gas. High pressures at 20–30 bars completely killed all life stages of *S. zeamais* and *T. castaneum* within a 5 min exposure. From our earlier study, application of carbon dioxide gas at cost effective pressure levels (4–8 bars) proved success in controlling *S. zeamais* without adverse effects on sensorial qualities of milled rice (Noomhorm et al., 2009). However *T. castaneum*, which is another important species of stored product insects found in rice mills, may respond to carbon dioxide gas differently. Additionally using of air as alternative to carbon dioxide gas in controlling the two species was attractive due to its ready availability. The aim of this study was to evaluate the potential of atmospheric and pressurized carbon dioxide gas and pressurized air for control of adults and immature stages of *S. zeamais* and *T. castaneum* in milled rice.

2. Materials and methods

2.1. Study overview

Three sets of experiments were carried out on *S. zeamais* and *T. castaneum*:

- Concentrations of 60, 80 and 100% carbon dioxide in air at atmospheric pressure were tested against adults and immatures.
- 100% carbon dioxide at 4, 6 and 8 bar pressure were tested against adults and immatures.
- Natural air at 4, 6 and 8 bar pressure were tested against adults only.

2.2. Preparation of insect samples

The aromatic rice cultivar, Kao Dawk Mali 105 (KDML 105), was obtained from Nakorn Sawan Province, Thailand. The paddy was dried and milled using horizontal rubber rollers and vertical abrasive rollers in a double-pass-whitening operation. The moisture content of fully milled rice was 13.4 ± 0.1% (wb). Adults of *S. zeamais* were collected from rice mills located in different locations in Thailand and cultured using coarse rice bran and milled rice (100:250 w/w). For tests requiring eggs, samples of milled rice (250 g) were infested with 50 adults for 2 days before treatment. Samples of milled rice (250 g) in which adults had laid eggs 18–20 or 25–27 days previously were used for tests on larvae, and pupae respectively.

Lots of 300 of adults of *T. castaneum* (Herbst) were reared in a mixture of rice bran and milled rice (100:250 w/w). After 2 days, 20–22 days and 27–29 days approximately 150 individuals of eggs, larvae and pupae, were put separately in 250 g of milled rice. For tests requiring adults, samples of milled rice (250 g) were infested with 150 adults. A small amount of rice bran (5% by weight) was mixed with the milled rice samples to improve the feeding conditions for the test insects.

Infested samples were placed individually in plastic containers (8 cm diameter × 9 cm high) each of which was covered with muslin cloth before being subjected to the treatment. Triplicates of infested rice samples containing insects at each life stage were prepared for each fumigation treatment and untreated control samples.

2.3. Set up of fumigation chambers

The apparatus used for treatment of insects was described by Noomhorm et al. (2009). There were three stainless steel cylindrical chambers (25 cm diameter × 50 cm high) each with sensors to measure the pressure inside the chambers using programmable logic control connected to solenoid valves through which carbon dioxide gas or air was supplied into the chambers. Boxes of infested rice samples were placed in the chambers and air was removed using a negative pressure pump (<1 bar). The chambers were filled with intended gas treatment to the required pre-set pressure.

Fumigation treatment was run three times simultaneously using three fumigation chambers. Each fumigation chamber was set up with one box for each of the four life stages (eggs, larvae, pupae, and adults) prepared for each exposure period. There were 4–18 boxes in each fumigation chamber.

2.4. Carbon dioxide fumigation at atmospheric pressure

For carbon dioxide fumigation at atmospheric pressure, carbon dioxide gas was purchased as pure gas at 100% (Praxair (Thailand) Co., Ltd) and 60 and 80% carbon dioxide with standard air as the balance (Thailand Industrial Gas, Thailand). Insect samples were placed inside the fumigation chambers and the air was evacuated. The carbon dioxide treatments were added to the chambers to the required pre-set pressure at 1 bar, which represented atmospheric pressure (1.01325 bar). Exposure times for adults in 60 and 80% carbon dioxide were 6, 12, 18, 24, 30, 36, 42, 48, 54, 60, 66 and 72 h, and in 100% carbon dioxide they were 3, 6, 9, 12, 15, 18, and 21 h. Exposure times for immature stages in 60 and 80% carbon dioxide were 10, 20, 30, 40, 50 and 60 h, and in 100% carbon dioxide they were 10, 20, 30, 40, 50, 60, 70, and 80 h.

2.5. Pressurized carbon dioxide fumigation

For pressurized carbon dioxide fumigation insect samples were put into fumigation chambers and then air was removed. Pure carbon dioxide gas at 100% (Praxair (Thailand) Co., Ltd) was supplied into the chambers in order to generate pressure of 4, 6 and 8 bars. Adults were exposed for 1, 2, 4, 6, and 8 h at 4 bars; and 0.5, 1,

1.5, 2, 2.5 and 3 h at 6 and 8 bars. Immature stages were exposure for 4, 6, 8, 10, 12 and 14 h at 4 bars; and 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6 and 6.5 h at 6 and 8 bars.

2.6. Pressurized air fumigation

To tests pressurized air alone, an air mixture of 76.5–80.5% N_2 and 19.5–23.5% O_2, was purchased from Praxair (Thailand) Co., Ltd. Air was added to the chambers to obtain pressures of 4, 6 and 8 bars. Only *S. zeamais* and *T. castaneum* adults were subjected to the treatment. Exposure times were 72, 144, 216 and 288 h at 4 bars; 24, 48, 72 and 96 h at 6 bars; and 15, 20, 25, 30, 35 and 40 h at 8 bars Mortality was assessed 1 day after treatment. Samples containing eggs, larvae and pupae were kept at laboratory temperature (30 ± 2 °C) for the required time for adult emergence. For samples originally containing eggs, larvae and pupae of *S. zeamais*, adult emergence was assessed after 45, 25 and 15 days respectively. The corresponding times for emergence of *T. castaneum* adults were 28, 21 and 14 days.

2.7. Data analysis

The experiment was performed in a completely randomized design and experimental triplicates were conducted at different times. Insect mortality data were analyzed with probit analysis using SPSS v.11.0 software (SPSS, 2001). Data for each life stage at each fumigation treatment were analyzed separately. Inputs to the probit analysis were dose (exposure time, h), number of insects treated and number of insects killed. Assessments were made on adults but as the number of eggs, larvae and pupae exposed to treatment were not known, these numbers were estimated from the untreated control samples (Weller and Morton, 2001; Beckett and Morton, 2003). These control samples had been handled and assessed in the same manner as the treated samples except for being fumigated. Estimated exposure times to achieve 50 and 99% mortality were calculated (Finney, 1964) among given life stages and fumigation treatments.

3. Results

3.1. Mortality of insects fumigated with carbon dioxide gas at atmospheric pressure

Efficacy of carbon dioxide gas at various concentrations (60, 80 and 100%) at atmospheric pressure against *S. zeamais* and *T. castaneum* is summarized in Table 1, where the values of LT_{50} and LT_{99} resulting from probit analysis are shown for the different life stages in each species. Carbon dioxide at 100% killed *S. zeamais* adults more rapidly ($LT_{99} = 16$ h) than at 60 and 80% ($LT_{99} = 21$ and 26 h, respectively) but the effect of carbon dioxide concentration against eggs, larvae or pupae was less obvious based on the frequent overlap of the fiducial limits for the LT values within stages. Based on the results for pupae, which were the most tolerant stage, an exposure period of up to 148 h at a carbon dioxide concentration of ≥60% would be needed for 99% or higher control of this species.

Carbon dioxide at 100% killed *T. castaneum* adults more rapidly ($LT_{99} = 10$ h) than at 60 and 80% ($LT_{99} = 38$ and 28 h, respectively). As was the case with *S. zeamais*, the effect of carbon dioxide concentration against eggs, larvae or pupae of was less obvious. Based on the results for pupae, which tended to be the most tolerant stage, an exposure period of up to 66 h at a carbon dioxide concentration of ≥60% to obtain 99% or higher control of this species.

3.2. Mortality of insects fumigated with pressurized carbon dioxide gas

Table 2 summarizes the efficacy of 100% carbon dioxide gas at various pressures (4, 6 and 8 bars) against *S. zeamais* and *T. castaneum*. These results, together with the results for the efficacy of 100% carbon dioxide gas at atmospheric pressure (Table 1), show that increasing pressure tended to reduce the LT_{50} and LT_{99} values for eggs, larvae, pupae and adults in both species. LT_{50} and LT_{99} decreased with levels of applied pressure. Pressure at 4 bars had less efficacy to kill all life stages of *S. zeamais* ($LT_{99} = 6.9$–29 h) than higher pressure at 6 and 8 bars ($LT_{99} = 1.3$–9.0 h and $LT_{99} = 2.0$–

Table 1
Response of four developmental stages of *Sitophilus zeamais* and *Tribolium castaneum* to carbon dioxide applied at atmospheric pressure with varying concentrations.

Insect species	Stage	CO_2 (%)	n[a]	Slope (±SE)	LT_{50}[b] (h) FL (95%)	LT_{99}[b] (h) FL (95%)
S. zeamais	Eggs[a]	60	522	0.111 (0.009)	17 (13–24)	38 (30–57)
		80	858	0.076 (0.004)	26 (21–31)	56 (48–73)
		100	2676	0.061 (0.002)	26 (23–29)	64 (58–73)
	Larvae[a]	60	750	0.066 (0.004)	28 (22–36)	64 (52–89)
		80	1542	0.054 (0.002)	35 (30–41)	78 (67–99)
		100	876	0.065 (0.003)	26 (15–37)	61 (48–91)
	Pupae[a]	60	1608	0.046 (0.002)	29 (22–37)	79 (64–111)
		80	552	0.056 (0.004)	24 (13–43)	66 (46–140)
		100	1998	0.022 (0.001)	43 (29–59)	148 (109–279)
	Adults	60	1800	0.206 (0.009)	15 (14–16)	26 (24–30)
		80	1500	0.319 (0.017)	13 (13–14)	21 (19–22)
		100	2400	0.324 (0.012)	8.4 (7.9–9.0)	16 (15–17)
T. castaneum	Eggs[a]	60	2544	0.156 (0.007)	23 (21–25)	38 (34–44)
		80	1872	0.139 (0.007)	16 (15–18)	33 (30–37)
		100	2228	0.285 (0.120)	22 (21–34)	31 (26–37)
	Larvae[a]	60	1434	0.076 (0.003)	29 (26–31)	59 (55–65)
		80	702	0.124 (0.008)	15 (11–20)	34 (27–47)
		100	1686	0.115 (0.008)	18 (16–19)	38 (34–42)
	Pupae[a]	60	2412	0.079 (0.003)	23 (21–25)	52 (49–57)
		80	2790	0.068 (0.002)	17 (14–20)	51 (45–59)
		100	2958	0.096 (0.008)	42 (38–45)	66 (64–70)
	Adults	60	2400	0.123 (0.005)	19 (15–24)	38 (31–55)
		80	1800	0.190 (0.008)	16 (15–17)	28 (26–31)
		100	1500	0.486 (0.023)	5.5 (5.1–6.0)	10 (9.4–12)

[a] $n = \Sigma N$ total number of insects for each experimental group.
[b] LT_{50} and LT_{99} are considered significantly different when the 95% fiducial limits (FL) fail to overlap.

Table 2
Response of four developmental stages of *Sitophilus zeamais* and *Tribolium castaneum* to pressurized carbon dioxide gas.

Insect species	Stage	Pressure (bars)	n[a]	Slope (±SE)	LT_{50}[b] (h) FL (95%)	LT_{99}[b] (h) FL (95%)
S. zeamais	Eggs[a]	4	2238	0.218 (0.009)	4.9 (2.6–6.3)	16 (13–22)
		6	2172	0.960 (0.044)	2.0 (1.7–2.1)	4.4 (4.1–4.7)
		8	810	1.120 (0.080)	2.5 (2.2–2.7)	4.6 (4.1–5.2)
	Larvae[a]	4	1284	0.072 (0.015)	4.4 (1.7–6.4)	15 (11–23)
		6	2370	0.369 (0.017)	2.7 (1.7–3.4)	9.0 (7.5–12)
		8	1209	1.211 (0.059)	1.4 (0.0–2.4)	3.3 (2.4–7.3)
	Pupae[a]	4	1608	0.118 (0.008)	9.2 (6.5–13)	29 (21–61)
		6	1092	0.718 (0.034)	2.3 (1.6–2.8)	5.5 (4.7–6.9)
		8	2076	0.771 (0.032)	1.7 (1.1–2.2)	4.8 (4.1–5.9)
	Adults	4	1500	0.756 (0.033)	3.8 (3.4–4.4)	6.9 (6.0–8.6)
		6	1500	2.789 (0.138)	0.4 (0.2–0.7)	1.3 (1.0–2.3)
		8	1500	3.123 (0.151)	1.3 (1.2–1.3)	2.0 (1.9–2.2)
T. castaneum	Eggs[a]	4	1548	0.940 (0.077)	4.4 (4.2–4.6)	6.9 (6.5–7.3)
		6	3060	1.862 (0.340)	2.2 (1.7–2.5)	3.5 (3.3–3.7)
		8	2118	4.231 (0.324)	0.6 (0.5–0.7)	1.2 (1.1–1.2)
	Larvae[a]	4	2226	0.378 (0.015)	4.6 (3.7–5.3)	11 (9.4–13)
		6	2088	0.947 (0.044)	2.1 (1.6–2.4)	4.5 (4.2–5.1)
		8	2274	3.489 (0.669)	0.9 (0.9–1.0)	1.5 (1.4–2.0)
	Pupae[a]	4	2766	0.200 (0.008)	3.8 (1.7–5.1)	15 (13–20)
		6	3198	0.629 (0.024)	2.1 (1.5–2.5)	5.8 (5.3–6.5)
		8	2820	3.077 (0.707)	1.1 (1.0–1.1)	1.8 (1.6–2.5)
	Adults	4	1500	0.906 (0.040)	2.9 (2.7–3.1)	5.4 (5.1–5.9)
		6	2100	1.529 (0.055)	1.4 (1.3–1.4)	2.9 (2.8–3.1)
		8	1500	1.650 (0.073)	0.8 (0.7–1.0)	2.3 (1.9–2.9)

[a] $n = \Sigma N$ total number of insects for each experimental group.
[b] LT_{50} and LT_{99} are considered significantly different when the 95% fiducial limits (FL) fail to overlap.

4.8 h respectively). Two high pressure levels showed similar effects on mortality of *S. zeamais* except in larvae stage, where LT_{99} were significantly different. At atmospheric pressure, exposure time required to obtain 99% mortality of all life stages of *S. zeamais* was ≤148 h (Table 1) whereas it was shorten to ≤29, ≤9.0 and ≤4.8 h when pure carbon dioxide was applied at 4, 6 and 8 bars respectively (Table 2). Adult was certainly the most susceptible stage to carbon dioxide applied at all pressure levels. Among immature stages, pupae were distinctively more tolerant than larvae and eggs when carbon dioxide applied at lowest pressure, at 4 bars. At 6 bars, however, the order of tolerance was larvae > pupae > eggs. But, at 8 bars, the difference in susceptibilities among immature stages was not clearly noticed.

As can be seen in Table 2, LT_{50} and LT_{99} for adult and immature stages of *T. castaneum* significantly decreased with levels of applied pressure. The highest pressure level at 8 bars had notable efficacy in killing *T. castaneum* (LT_{99} = 1.2–2.3 h) when compared to 4 and 6 bars (LT_{99} = 5.4–15 h and LT_{99} = 2.9–5.8 h respectively). LT_{99} for all life stages of this specie was ≤66 h using pure carbon dioxide at atmospheric pressure but they reduced to ≤15, ≤5.8 and ≤2.3 h respectively with increasing pressure to 4, 6 and 8 bars. Pupae of *T. castaneum* were more tolerant than larvae and eggs to pressurized carbon dioxide. Adult stage of *T. castaneum* was the most susceptible at low pressure levels (4 and 6 bars), however it was the most tolerant at the highest pressure at 8 bars. When all life stages of two insect species were compared, *T. castaneum* was

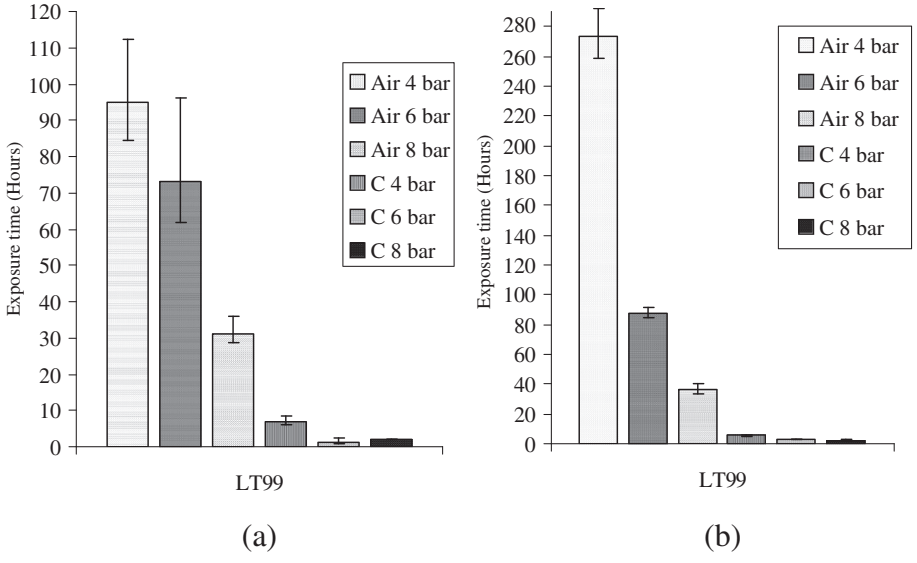

Fig. 1. Exposure time (h) required to obtain 99% mortality (LT_{99}) of adults of (a) *S. zeamais* and (b) *Tribolium castaneum* after exposure to air (Air) or pure carbon dioxide (C) at three pressure levels.

less tolerant to pressurized carbon dioxide than *S. zeamais*. Adults of *T. castaneum*, however, were more tolerant to pressurized carbon dioxide gas than adults of *S. zeamais*.

3.3. Mortality of insects fumigated with pressurized air

Pressurized air had lower efficacy against adults of *S. zeamais* and *T. castaneum* compared to pressurized 100% carbon dioxide gas (Fig. 1). For pressurized air, LT_{99} values of 95, 73 and 31 h were required to kill adults of *S. zeamais* at 4, 6 and 8 bars respectively; compared to 6.9, 1.3 and 2.0 h when using pressurized carbon dioxide (Table 2). For *T. castaneum*, the corresponding results were 273, 88 and 37 h for pressurized air, and 5.4, 2.9 and 2.3 h for pressurized carbon dioxide at 4, 6 and 8 bars respectively. LT_{99} of adults of the two insect species exposed to pressurized air were also longer than those treated with pure carbon dioxide gas at atmospheric pressure. They were 16 h and 10 h for *S. zeamais* and *T. castaneum* respectively (Table 1).

4. Discussion

Carbon dioxide gas has been found to be toxic to insects in stored grain and this is the case with the two species investigated in this study, i.e. *S. zeamais* and *T. castaneum*. Stored grain insects respond to carbon dioxide differently. As mentioned in our previous work (Noomhorm et al., 2009), the LT_{99} of *S. zeamais* after exposure to 100% carbon dioxide (0.65–6.17 days) was much lower than those of *S. oryzae* ranging from 4.22 to 22.80 days (Annis and Morton, 1997), which could be due to either natural differences between these species or the different techniques used to add carbon dioxide gas. In the current study, removal of air with a vacuum pump, and adding gas to a set pressure level (1 bar) or releasing gas suddenly may have injured the tested insects.

There tended to be no difference between the efficacy of 60, 80 or 100% carbon dioxide within the egg, larval or pupal stages; however carbon dioxide gas mixed with air was likely more toxic to *S. zeamais* than pure carbon dioxide gas. A two times longer exposure time was required to kill all life stages of *S. zeamais* when using 100% carbon dioxide compared to 60 and 80%. This might be due to the non-monotonic insect mortality with respect to carbon dioxide concentration. Toxicity of carbon dioxide and toxicity of anoxia increased with carbon dioxide concentration so that increasing of mortality was expected. However, antagonism of low oxygen to toxicity of carbon dioxide was observed, which resulted in decreasing of mortality with increasing carbon dioxide concentration. The similar findings were presented by researchers who studied other weevil species such as *S. granarius* and *S. oryzae* (Lindgren and Vincent, 1970; Annis and Morton, 1997). Moreover, this study proved that the adult stage of *S. zeamais* was the most susceptible stage in its life cycle to carbon dioxide gas exposure. Pupae tended to be the most tolerant stage, which was similar to the reports for *S. oryzae* (Lindgren and Vincent, 1970; Annis and Morton, 1997).

There were no noticeable differences in the effect of carbon dioxide against eggs, larvae or pupae of *T. castaneum*. For adult stage, the outcome of this study was similar to other researchers, who reported more prominent effects on mortality of *T. castaneum* adults when higher concentration of carbon dioxide gas was applied (Harein and Press, 1968; AliNiazee, 1971; Jay and Pearman, 1971). Likewise, exposure times reported in their studies were comparatively long. More than 48 h was required to kill all adults of *T. castaneum* when pure carbon dioxide gas was purged in simulated peanut storage (Press and Harein, 1967), whereas only 18 h was reported when exposing gas directly to insects in the empty containers (AliNiazee, 1971). The differences of mortality responses to carbon dioxide gas might be due to gas applying techniques, employing temperatures, age of tested insects and gas penetrability. However, similar findings on order of tolerance of *T. castaneum* exposed to pure carbon dioxide were presented by AliNiazee (1971). Adults of *T. castaneum*, as the active stage, were found to be the most susceptible to carbon dioxide, whereas pupae, as the dormant stage, were the most tolerant to carbon dioxide.

The response of insects to high pressure and conventional carbon dioxide treatments was found to be different. Two ways in terms of physiological and physical actions to achieve insect mortality by high pressure carbon dioxide were suggested by many researchers (Caliboso et al., 1994; Nakakita and Kawashima, 1994; Reichmuth and Wohlgemuth, 1994). The physiological action of carbon dioxide at high concentration seemed to have less influence on the mortality than the physical action of high pressure gas in tissues of the insects. Carbon dioxide actually dissolves in the body of insects. Under high pressure, gas expands and then rapidly equilibrates to atmospheric pressure so that severe damage to the insect body with loss of their integument could occur. Thus increasing of applied pressure reduced exposure times to obtain required mortality. This was in agreement with our previous study (Noomhorm et al., 2009) and present study as well that shortest LT_{99} of all life stages of *S. zeamais* and *T. castaneum* was reported when using the highest pressure, at 8 bars.

The two species of insect responded differently to high pressure carbon dioxide. When all life stages of the insects are considered, *S. zeamais* seems to be more tolerant than *T. castaneum*, but adults of *T. castaneum* were more tolerant than those of *S. zeamais*. This was confirmed by Nakakita and Kawashima (1994) who tested adults of four species and reported that *T. castaneum* was the most tolerant, followed by *S. zeamais*, *Rhyzopertha dominica*, and *L. serricorne*.

Besides species differences, differences in response was also found among the different life cycle stages of the insects. For *S. zeamais*, adults were confirmed as the most susceptible life stage (Nakakita and Kawashima, 1994; Noomhorm et al., 2009). However, the findings from our earlier work (Noomhorm et al., 2009) and current study were slightly different in that, among immature stages, eggs were not the most tolerant stage to high pressure carbon dioxide. The order of resistance was similar to that obtained from carbon dioxide treatment at atmospheric pressure. This phenomenon might be because the pressure applied in our studies was comparatively low (up to 8 bars) compared to their studies which used pressure up to 30 bars to kill *S. zeamais*. The level of pressure played had a significant impact on the response of different insect life stages to carbon dioxide gas. At low pressure levels used in this study (up to 8 bars), the order of resistance among immature stages of *T. castaneum* was pupae > larvae > eggs, whereas, it was eggs > larvae > pupae in the report of Caliboso et al. (1994) who used pressure up to 30 bars. It was observed that eggs were also more susceptible than larvae and pupae when pure carbon dioxide gas was applied at atmospheric level. Influences of physiological action and physical action of carbon dioxide on insect mortality might be different among pressure levels.

Failure to kill *S. zeamais* and *T. castaneum* adults occurred when pressurized air was used instead of carbon dioxide gas. A similar finding was reported when helium was applied and had almost no effect on mortality of *S. zeamais* adults even at the high pressure at 70 bars (Nakakita and Kawashima, 1994). Solubility of gas in hemolymph of insects, which is composed mainly of water, is an important factor in efficacy of pressurized carbon dioxide against insects because dissolved gas expands as the gas is suddenly released to the atmosphere so that tissues of insects are destroyed. Likewise quick evaporation of gas from the liquid in insect bodies

leads to lesions of cell membranes (Reichmuth and Wohlgemuth, 1994). Efficacy was enhanced at high pressure levels. From Henry's law, at constant temperature, the amount of gas dissolved in water is directly proportional to the partial pressure of the gas. So higher solubility of carbon dioxide gas under high pressure may have increased efficacy. Pressurized air is composed mainly of nitrogen and oxygen gas. Nitrogen and oxygen have lower solubility compared to carbon dioxide. At 25 °C, the mole fraction solubility of nitrogen, oxygen and carbon dioxide is 1.183×0^{-5}, 2.293×10^{-5} and 6.15×10^{-4} respectively (Gevantman, 1988).

Carbon dioxide gas was found to be toxic to *S. zeamais* and *T. castaneum*, however fumigation with carbon dioxide gas at atmospheric pressure required exceedingly long exposure time ($\geq$148 h and $\geq$66 h) to kill all live stages of both insect species. Exposure times were reduced approximately 30 times when using pressurized carbon dioxide at 8 bars. Only a few hours were needed ($\geq$4.8 h and $\geq$2.3 h) in order to kill *S. zeamais* and *T. castaneum*, respectively. Application of carbon dioxide gas at this pressure level is practical due to using cost effective equipment and at ease operation. Air could not be used instead of carbon dioxide gas. Gas with highly soluble in liquid in insect bodies and more toxic than carbon dioxide gas was recommended in order to shorten exposure time.

Acknowledgments

This research was financially supported by Thailand Research Fund and Rice Engineering Supply Co., Ltd., Thailand.

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