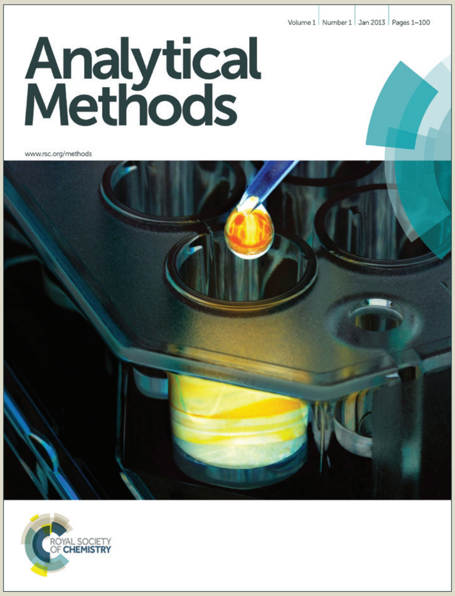
Analytical Methods



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Non-destructive evaluation of total volatile basic nitrogen (TVB-N) and K-values in fish using colorimetric sensor array

[1]Xingyi Huang*, [1]Riqin Lv, [1]Liya Yao, [1]Chao Guan, [1]Fangkai Han, [2]Ernest Teye
[1]School of Food and Biological Engineering, Jiangsu University,
Xuefu Road 301, Zhenjiang 212013, Jiangsu, P. R. China
[2]School of Agriculture, Department of Agricultural Engineering,
University of Cape Coast, Cape Coast, Ghana
*Corresponding author: Xingyi Huang, Email: h_xingyi@163.com
Tel: +86-51188792368, Fax: +86-51188797308

Abstract

For rapid evaluation of fish freshness, a colorimetric sensor array has been developed for the sensitive detection to measure simultaneously TVB-N and K value of fish during its storage period. Silver carps were taken as fish samples which were stored at constant temperature of 4℃during experiment period. 10 kinds of porphrin compounds and 6 pH indicators were selected as chromogenic materials in this experiment according to the previous study and the theoretical research. For comparison, total volatile basic nitrogen (TVB-N) values of fishes were tested by conventional chemical method, and the K-values were measured using High Performance Liquid Chromatography (HPLC). As sensing materials used in the sensor array were chromogenic, the color of the sensor array changed when reacting with odor emitted by fish sample. The color change profiles of the sensor array before and after exposure to the odor of each sample were got using image processing method. And color features were extracted to be analyzed using principal component analysis (PCA), linear discriminant analysis (LDA). The relationship between these analysis results and the TVB-N values and K-values obtained by conventional methods were established using support vector regression (SVR). And therefore models were set up for rapid prediction of TVB-N values and K-values, respectively. For the SVR model of TVB-N content and K-values, calibration correlation coefficient (R_{tr}) was 0.8564 and 0.8712, and the root mean square error of calibration (RMSEC) was 4.2177 and 0.06127, respectively. It is feasible to predict TVB-N values and K-values according to experiment results of colorimetric sensor array. The results indicated that the novel method based on colorimetric sensor array developed provide a feasible way for rapid and nondestructive evaluation of fish freshness.

Keywords: fish freshness; colorimetric sensor array; total volatile basic nitrogen;

K-values

1.0 Introduction

For thousands of years, the fish product is deeply favored by the people in many countries as it is rich in protein and of delicious taste. However, fish also has the disadvantage of comparatively short shelf life (Yao, Zhang et al. 2011). Public attention to fish quality and safety has increased significantly in recent decades, due in part to changes in consumer behavior and the gradually increasing consumption of fish. The safety of fish is of course important to consumer. Loss of freshness and spoilage of fish are rather complex processes and various factors such as microbiology, enzyme influence the spoilage pattern. In fact, the development of reliable methods to assess fish freshness and to evaluate quality criteria has been researched for many years (Di Natale, Olafsdottir et al. 2001). The traditional methods for determining fish freshness are based on physical, chemical, microbiological measurement and human sensory evaluation (Olafsdottir, Nesvadba et al. 2004). The human array evaluation is an immediate assessment of quality and can be done with no damage to fish. On the other hand, it is not easy to keep evaluation results consistent among different assessments, because the evaluation score is subjective. Other methods including microbiological measurement and chemical methods are limited in the laboratory and not convenient on-site because they are time consuming and destructive detection. Instrumental method independent of the subjective opinion of human judge is needed to meet the demand for quality measurement in the fish industry.

Recently, plentiful rapid instrumental analytical techniques also have been developed for the sensitive detection of the spoilage of fish, such as near infrared (NIR) spectroscopy (Armenta, Coelho et al. 2006), electronic tongue (Han, Huang et al. 2014), and electronic nose (Limbo, Sinelli et al. 2009). Odor is one of the most important parameters for evaluating the freshness of food. Once the fish dies, a very large amount of microbes of fish breed produces volatile organs (VOCs), which are closely related to the freshness of fish. Each product has a characteristic profile of volatile compounds and therefore its own characteristic odor. Likewise, spoilage will result in a different but still characteristic profile of volatile compounds in the same product. Kenneth has previously reported the colorimetric array detection of a wide range of odorants, using a family of metalloporphyrins immobilized on reverse-phase silica and on hydrophobic membranes (Suslick, Rakow et al. 2004). Gas sensor array, a new concept of electronic nose, compared with traditional electronic nose, the technology has the advantages of high precision, wide range and it is not influenced by humidity (Huang, Xin et al. 2009). It transformed olfactory information into visual information by color's change after pigments reacted with volatile compounds and chemometrics analysis was carried out on the visual information (Zou and Zhao 2008). In the past decades, many kinds of colorimetric sensors have been developed to monitoring food freshness based on different chromogenic materials. Artificial olfaction system has been applied to the analysis of beer (Zhang, Bailey et al. 2006)and soft drink (Zhang and Suslick 2007, Chen, Liu et al. 2013), classification of tea (Chen, Liu et al. 2013), evaluation of pork freshness, and monitor fish packaging (Kuswandi, Restyana et al. 2012). Our previous research also has applied colorimetric sensor array to evaluate fish freshness (Huang, Xin et al. 2011). Researches mentioned above show that an artificial olfactory technique based on colorimetric sensor array has huge potential in the analysis of food, according to their volatile organic compounds (VOCs).

Silver carps were taken as fish samples in this study. Because the silver carp is different from other fish detected before in physical size and composition, it is necessary to optimize the test parameters including the way of gas sampling and array keeping. In previous study, volatile organic compounds were acquired by applying carrier gas to bring the top gas of sample to reaction chamber and the colorimetric sensor array was kept in a nitrogen atmosphere. In this study, volatile organic compounds were acquired by free-diffusion in reaction chamber and each array was kept in a Hermetic bag.

This study is therefore intended to develop an E-nose system of fish freshness based on TVB-N and K-values, regarding the silver carp which has the largest products and sales as detection object. The detailed work was arranged as follows: (1) fabricating an E-nose system of colorimetric sensor arrays (2) determining chemical indicators (3) using Fisher linear discriminant (Fisher LDA) for sensors data classification, and support vector regression (SVR) models for prediction of quality parameters (TVB-N and K-values).

2.0 Materials and methods

2.1. Samples preparation

Silver carp fishes (Hypophthalmichthys molitrix) from a local aquaculture farm of average size of 950g were adopted in the experiment. Before the tests, all the fishes were cleaned, weighted and were individually placed in freshness protection packages.

The samples were then stored in a refrigerator at 4℃. The chemical and E-nose analyses of the samples were conducted every day and lasted for 12 days.

2.2. Colorimetric sensor array

Fabricating a colorimetric sensor array is often based on two crucial requirements: (1) every chemically responsive dye must contain a center interacted strongly with analytes, and (2) the interaction center must be strongly coupled to an intense chromophore. The required dye classes include: (i) Lewis acid/base dyes (i.e., metal ion containing dyes), (ii) Brønsted acidic or basic dyes (i.e., pH indicators), and (iii) dyes with large permanent dipoles (i.e., zwitterionic solvatochromic dyes) (Janzen, Ponder et al. 2006)Porphyrins and their metal complexes are a natural choice for recognition of analytes with Lewis acid/base capabilities. Metalloporphyrins are nearly ideal for the detection of metal-ligating vapors because of their open coordination sites for axial ligation, their large spectral shifts upon ligand binding, and their intense coloration. Common pH indicator dyes change color in response to changes in the proton (Brønsted) acidity or basicity of their environment (Suslick, Rakow et al. 2004). Colorimetric sensor array was made by printing the chemical responsive dyes on the hydrophbic plate, silicagel plate which were used as the plate of the sensor array in the experiment according to the experiment conducted before (Huang, Xin et al. 2011). To choose optimum chromogenic sensing materials, the characteristic gases of silver carps were determined by Gas Chromatography-Mass Spectrometer (GC-MS). Combined with the theoretical research (Gu, Huang et al. 2014, Gu, Huang et al. 2014, Huang, Gu et al. 2014), ten porphyrin compounds and six pH indicators were used as sensing pigments. The colorimetric sensor array was prepared by placing each material onto the silicagel plate to obtain a 4×4 array. The ten porphyrins or metalloporphyrins materials were purchased from Sigma Chemical (USA), including:

5, 10, 15, 20-Tetraphenyl-21H, 23H-porphine manganese (III) chloride
Zinc 2, 3, 9, 10, 16, 17, 23, 4-octakis-(octyloxy)-29H, 31H-phthalocyanine
2, 3, 7, 8, 12, 13, 17, 18-Octaethyl-21H, 23H-porphine manganese (III) chloride
5, 10, 15, 20-Tetraphenyl-21H, 23H-porphine copper (II)
5, 10, 15, 20-Tetraphenyl-21H, 23H-porphine zinc, (low chlorin, synthetic)
5, 10, 15, 20-Tetrakis (pentafluorophenyl)-21H, 23H-porphyrin iron (III) chloride
5, 10, 15, 20-Tetraphenyl-21H, 23H-porphine

5, 10, 15, 20-Tetraphenyl-21H, 23H-porphine iron (III) chloride

5, 10, 15, 20-Tetraphenyl-21H, 23H-porphine cobalt (II)

5, 10, 15, 20-Tetraphenyl-21H, 23H-porphine nickel (II)

Six types of pH indicators were obtained from Sinopharm Chemical Reagent Co. Ltd., including: (1) Bromocresol Green, (2) Bromothymol Blue, (3) Methyl Red, (4) Neutral Red, (5) Bromocresol Purple, and (6) Cresol Red.

The detailed steps of fabricating colorimetric sensor were arranged as follow: (1) dissolve the porphyrins and metalloporphyrin materials in chloroform, and dissolve the pH indicators in ethanol and; (2) 16 kinds of pigments solution of 2 mg/mL were eventually obtained after ultrasound for hours;(3) Each pigments solution was printed on the plate using 0.1 μ L microcapillary pipettes, constructing a 4×4 sensor array; (4) dried the sensor arrays in fume hood, and kept each of them in a Hermetic bag before use.

2.3. E-nose system and data acquisition

A fully functional prototype device for fish was constructed and the schematic diagram is shown in Fig 1. The enriched VOCs from samples were collected by free diffusion. In this experiment, the details were as follows: (1) an initial image was acquired before a sensor was used by HP scanjet 4890 flatbed scanner. (2) A complete fish was put in the sample room, and then the prepared sensor array chips were sticked on the sealing cap, with the surface containing pigments facing down. And (3) a final image was acquired after chubs' headspace volatile gas reacted with the chromogenic materials on the sensor array for 2 minutes.

The response of each of the sixteen pigments in the sensor was represented by the red, green and blue values, which resulted in a 48 dimensional vector (16dyes×3 color components RGB). This visual representation extracted from differences of images before and after experiment was obtained in the environment of OPENCV. All the data of RGB components were used in statistical and quantitative analyses and subsequent pattern recognition.

2.4. TVB-N and K-values

The total volatile basic nitrogen is one of the main physical and chemical experiments to evaluate the freshness of fish. The total volatile basic nitrogen of each fish samples was measured with micro Kjeldahl method according to SC/T3032-2007 (South China Sea fisheries research institute 2007). A 10g portion of sample was taken aseptically and homogenized after adding 90mL perchloric acid solution. It was filtered and the filtrate was directly distillated, and then titrated.

The ATP-related compounds, consisting of inosine-50-monophosphate (IMP), inosine (Ino), and hypoxanthine (Hx), were extracted by perchloric acid and separated and quantified by high-performance liquid chromatography. The analysis was conducted on a high-performance liquid chromatography (Ultimate 3000 Germany DIONEX Company). Separations were made on a reverse-phase Ultrabase C184.6×150 mm, with an internal particle diameter of 5μm (Shimadzu inertsustain). ATP, ADP, AMP, IMP, HxR and Hx were quantified according to the external standard method using calibration curves of the peak area of compound versus the compound concentration under identical chromatographic conditions. K-values were then calculated according to Eq (1).

$$\mathrm{K}(\%) = \frac{100(\mathrm{HxR}+\mathrm{Hx})}{\mathrm{ATP}+\mathrm{ADP}+\mathrm{AMP}+\mathrm{IMP}+\mathrm{HxR}+\mathrm{Hx}} \qquad (1)$$

Where ATP, ADP, AMP, IMP, HxR, Hx represent the concentration (μmol/g) of adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, inosine monophosphate, inosine, hypoxanthine.

2.5. Statistical method

In the data processing, Fisher linear discriminant (Fisher LDA) and support vector regression (SVR) were used for statistical analysis. All the analyses were performed using Matlab 2012a (Mathworks, Natick, MA) in windows 7.

3.0 Results and discussions

3.1. Physico-chemical analyses

3.1.1 Measurement of TVB-N by conventional methods

The initial TVB-N content of the fish (Fig. 2(a)) was 12.6mg/100g (at Day 1) and the final TVB-N was up to 45.7mg/100g, which could be related to the activity of spoilage bacteria and endogenous activities. TVB-N of silver carp fish remained stable up to day 4, and slightly increased during day 4~day 7 and strongly increased thereafter. In the present study, an arbitrary value for TVB-N of 13mg/100g was taken for the upper acceptability limit of fresh fish and 20mg/100g was used to mark the value of not edible(South China Sea fisheries research institute 2007). The two values were exceeded on day 2 and day 8.

3.1.2 ATP related compounds and K-values measured by HPLC

The ATP in the fish muscle would degrade in the way of ATP → ADP → AMP

→ IMP → HxR → Hx after fish was died. From Fig. 2(b), the changes of inosine 5'-monophosphate (IMP) and hypoxanthine (HX) were depicted during a cold storage of 12 days. The IMP levels at the beginning of the study were at the average of 5 μ mol/g. The concentration of this metabolite progressively decreased throughout storage with levels under 1.24 μ mol/g at the end of this study. HX values increased slowly and remained at low levels during the 12 days of this study.

The K-values (Fig. 2(c)), which measures the extension of IMP degradation, was worked out to reduce this variability. The K-values increased during storage from values 10%~52%. The K-values rises faster in the first three days because when it becomes stiff, ATP decomposes quickly, K-values increase rapidly and during this time, protein decomposes slowly. Compared with TVB-N, K-values accurately reflect the changes of freshness of the fish in the early time. It is generally believed that the K-values of fresh fish must be 10% (Yang, Xue et al. 2007, Yao, Zhang et al. 2011), the K-values of fresh goods such as sashimi in Japan 20%, the K-values of the second freshness is 20%～40% and the K-values of the fish which decompose at the first time is 60%～80%. From Fig. 2(c), it can be seen that in the first day of silver carp's storage, its freshness was close to 10%, 18 samples had K-values that are about 10%, and the average value was 10.64%; In the second day, the average of the K-values was up to 19.67% and most of the silver carps fish were still fresh; in the third to the seventh day, the K-values of the samples were in the range of 20%～40% and the silver carps was considered as second level freshness; in the twelfth day, the decay was very obvious.

3.2. The change of visual sensor array response

Difference maps were obtained by the difference between the red, green and blue (RGB) of each sensing material before and after experiment. The difference in the vector is shown in Fig. 3 as a map of the absolute values of color changes. Fig. 3 clearly shows the presence of characteristic color fingerprints for each day, thus confirming the possibility of using this array to monitor fish spoilage. With the changes of the species and the composition of the volatile gases during silver carps' spoilage, almost all the dyes' color changed and the changes was enhanced strength. During the early time of cold storage, the silver carps were fresh and there was less volatile component, most of which were hydrocarbons and short C chain alcohols (Dai, Huang et al. 2012). They could only generate non-specific adsorption with the porphyrin-based compound and the sensor response was slight. The response of the pH indicators was more evident than porphyrin-based compound, which were mainly due to the action of a small amount of acid gases. During the medium-term of storage, silver carps were in the second level freshness and the fish began to decompose, producing volatile gases containing of nitrogen, sulfur and so on. As the time of cold storage prolonging, the concentration of some species of the volatile gases in fish began to decrease, while the concentration of the nitrogen-containing and sulfur-containing gases continued to rise. This phenomenon led to the porphyrins response which became more obvious than the pH indicators and the overall color of array was obviously different from that in early times.

3.3. Correlation between colorimetric sensor array method and conventional method

PCA was used for classification of the samples from three types of fish in this work. PCA is a linear, unsupervised and pattern recognition technique used for analyzing, classifying and reducing the dimensionality of numerical datasets in a multivariate problem (Zhao and Lin 2012). It can transform original variables into a few new variables, known as principal compounds (PCs). To visualize the cluster trends of these samples, a 3-dimensional scatter plot (see Fig. 4(a)) was constructed using PC1, PC2 and PC3. Herein, the top three PCs accounted for 82.53% of total variances in raw data. The first six factors explained 91.2% of total variance. But PCA is an unsupervised technique and hence LDA was employed. LDA is probably the most frequently used supervised pattern recognition. The optimal transformation in classical LDA is obtained by minimizing the within-class distance and maximizing the between-class distance simultaneously, thus achieving maximum class discrimination (Zhao and Lin 2012). Similar to PCA, a 2-dimentional plot was constructed using DF1 and DF2 in this work, and all samples cluster can be visualized in this plot as shown in Fig. 4(b). DF1 and DF2 can explain almost100% of total variances. According to TVB-N determination results, the output of the LDA model can be defined as three levels--- fresh (group-1), not fresh (group-2), not edible (group-3). A total of 216 samples were ranked in order of spoilage time. All samples were divided into two sets, one sample taken out from every six samples was assigned to the test set and the other five samples were assigned to the train set. As a result the train set has 180 samples and the test set has 36 samples. In test set, 6 samples were classified in error and in train set, 28 samples were classified in error. The accuracy of test set and train set were 83.33% and 84.44%.

Since the PCA and LDA analyses of the colorimetric measurements and physico-chemical values classified the samples into similar groups, the SVR statistical tool was used to predict physicochemical values from the optoelectronic array data. SVR is a novel learning method that has solid theoretical basis and requires only small amount of sample. Different from present statistical approaches, it doesn't involve probability measure, law of large numbers and so on. In essence, it avoids traditional process from induction to deduction, realizing the efficient transductive inference from training sample to forecast sample, which greatly simplified the regression process. A rapid increase in TVB-N content with time was accompanied by parallel in the formation of K-values and the color changes of the colorimetric sensor array. The input values were the first six factors from PCA. To develop a TVB-N and K value prediction model, the samples were divided into two sets as before. The experimental values versus the values predicted by the SVR statistical models for both TVB-N content and K-values are shown in Fig. 5. Fig. 5 (a) shows the SVR-predicted TVB-N versus the actual TVB-N, and Fig. 5 (b) shows the SVR-predicted K-values versus the actual K-values. A preliminary evaluation of the accuracy of the created prediction model can be made by visually inspecting the difference between the measured and predicted values. However, a more rigorous analysis was achieved by linearly or nonlinearly fitting the experimental points. The correlation coefficients relate to accuracy in the prediction, whereas the root mean square error (RMSEC) relates to the SVR model’s precision (Zhao and Lin 2012). Good correlations between the gas sensor array results and both TVB-N analysis and K value analysis were obtained. In the model, for TVB-N, correlation coefficients (r2) of 0.733and 0.627 in train set and test set are obtained, while for K value, correlation coefficients (r2) of 0.759 and 0.596 in train set and test set are obtained, respectively. Moreover, values of 4.2177, 4.9563 and 0.06127, 0.0789 were found for RMSEC for the train set and RMSEP for the test set of TVB-N content and K-values, respectively. In this way, the ability of the electronic nose to predict TVB-N and K value was investigated for the fish samples. Due to the good correlation between the colorimetric sensor array and TVB-N and K value, the colorimetric sensor array could be used as a rapid non-destructive testing for TVB-N and K Value of Silver Carps and also could be used for grouping fish freshness.

4.0 Conclusions

A colorimetric sensor array for monitoring fish spoilage has been developed. The array consisted of sixteen chromogenic sensing materials including ten porphrin compounds and six pH indicators. The detection of TVB-N content and K-value was also carried out. All fish were classified into fresh, not fresh, not edible according to the test. PCA and Fisher LDA analysis were employed to analyze the chromogenic data from sensor arrays. The images obtained from PCA and Fisher LDA analysis both clearly showed three main clusters that correlated quite well with the classification from chemical analysis. A good correlation was obtained by SVR statistical analysis between the gas sensor array response and both TVB-N content and K value. Compared with the commercial studies, the colorimetric sensor array is just a chip, with no space occupation and simple to make with 3 minutes only. Besides, it is cheap and easy to detect products with no destruction quickly. And considering the relation between the chromogenic data and fish decay, the results indicated that the novel method based on colorimetric sensor array developed provide a feasible way for rapid and nondestructive evaluation of fish freshness.

Acknowledgements

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Figures

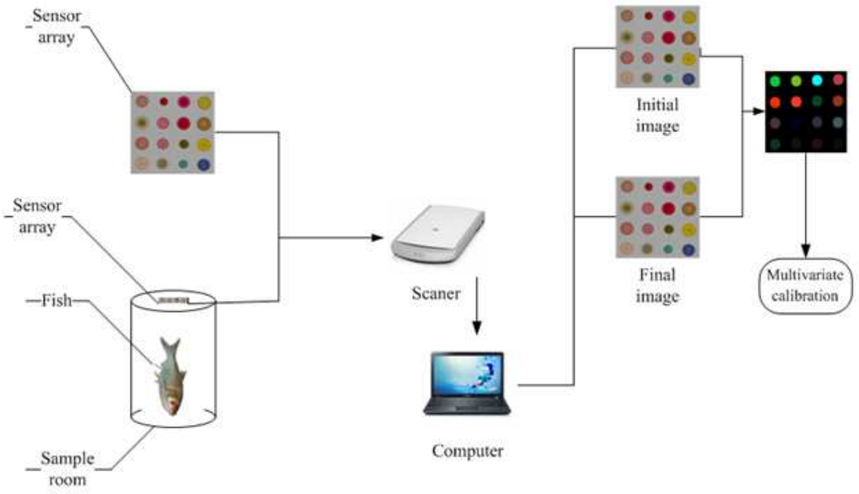

Fig.1. Schematic diagram of artificial olfaction system for fish freshness

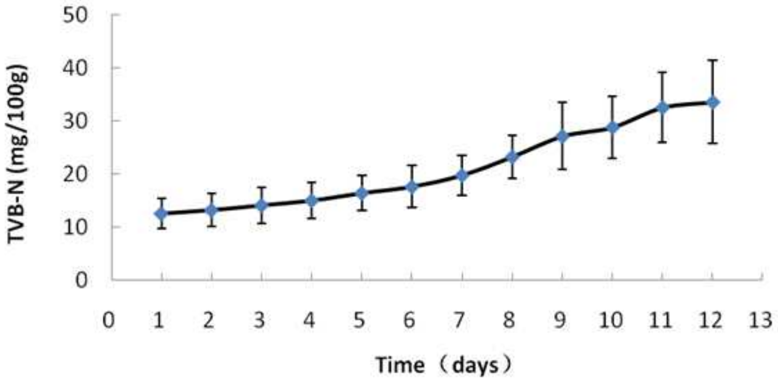

Fig. 2 (a) Changes of TVB-N content of silver carp during the cold storage

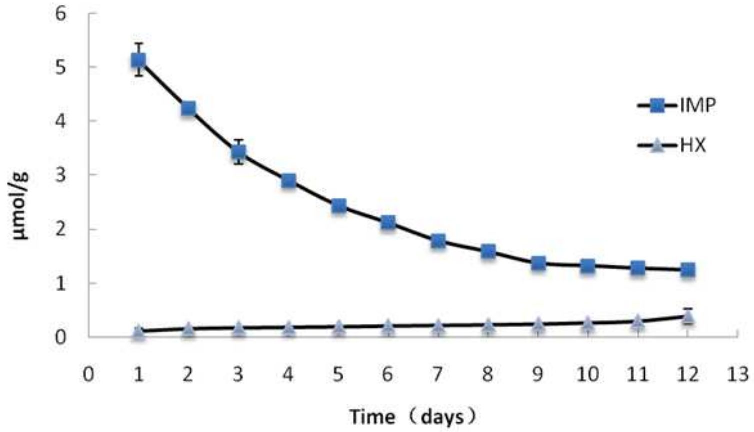

Fig. 2 (b) Changes of IMP and HX content of silver carp during the cold storage

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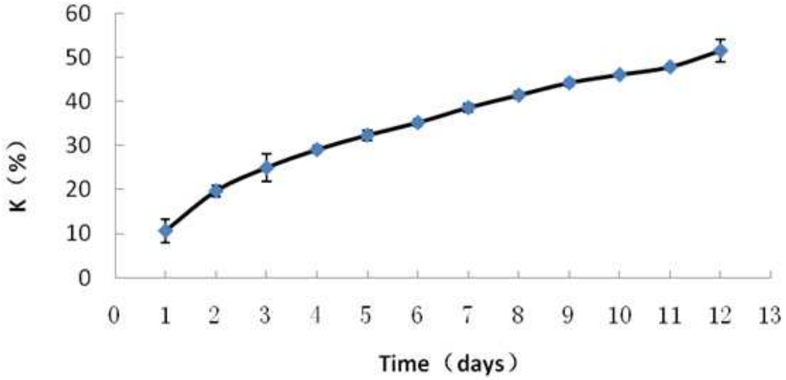

Fig. 2 (c) Changes of K value of silver carp during the cold storage

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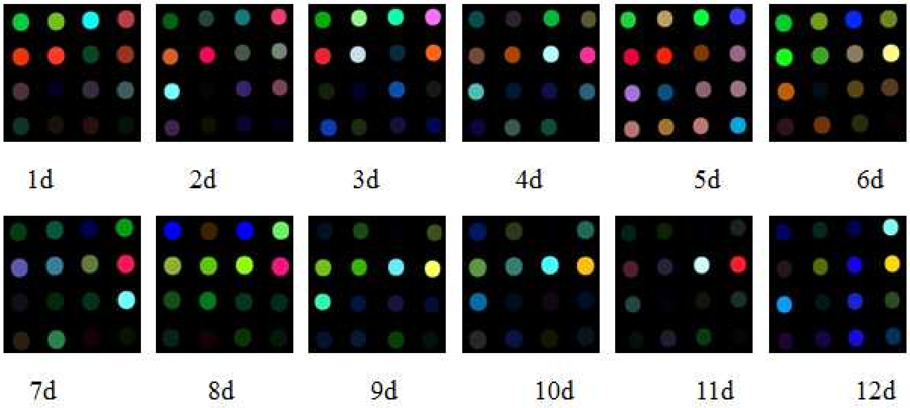

Fig.3 Characteristic images of silver carp during the cold storage

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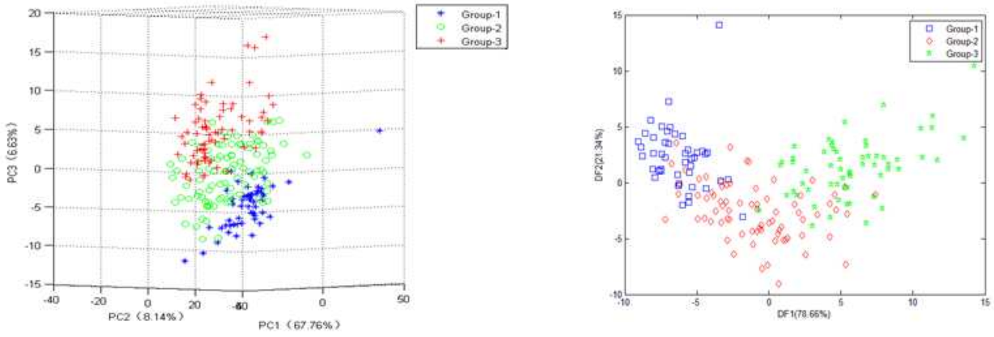

(a) (b)

Fig.4. Classification results achieved by PCA (a) and LDA (b)

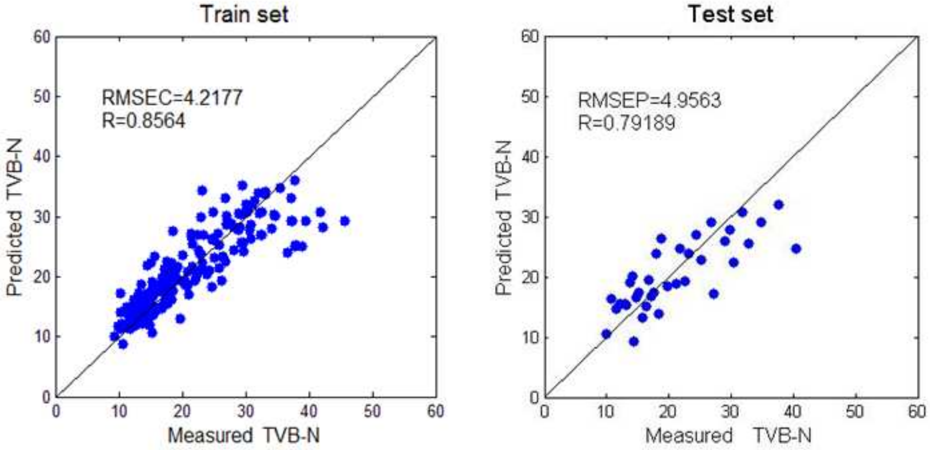

Fig.5 (a) Correlation between measured TVB-N and prediction value by SVR

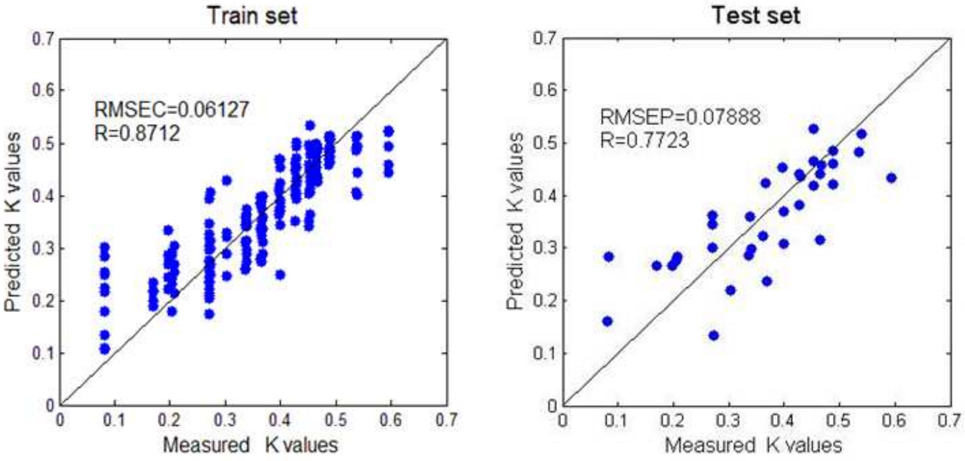

Fig.5 (b) Correlation between measured K value and prediction value by SVR