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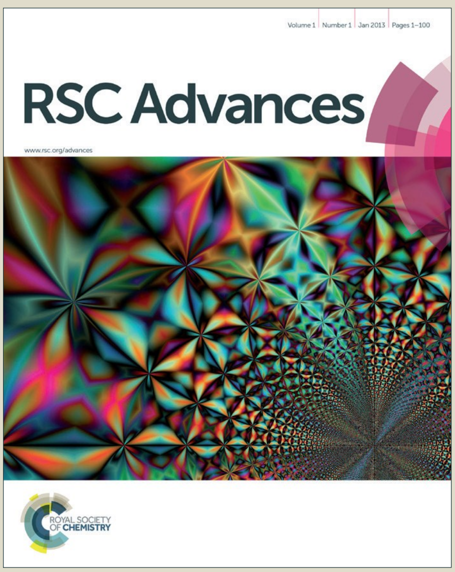

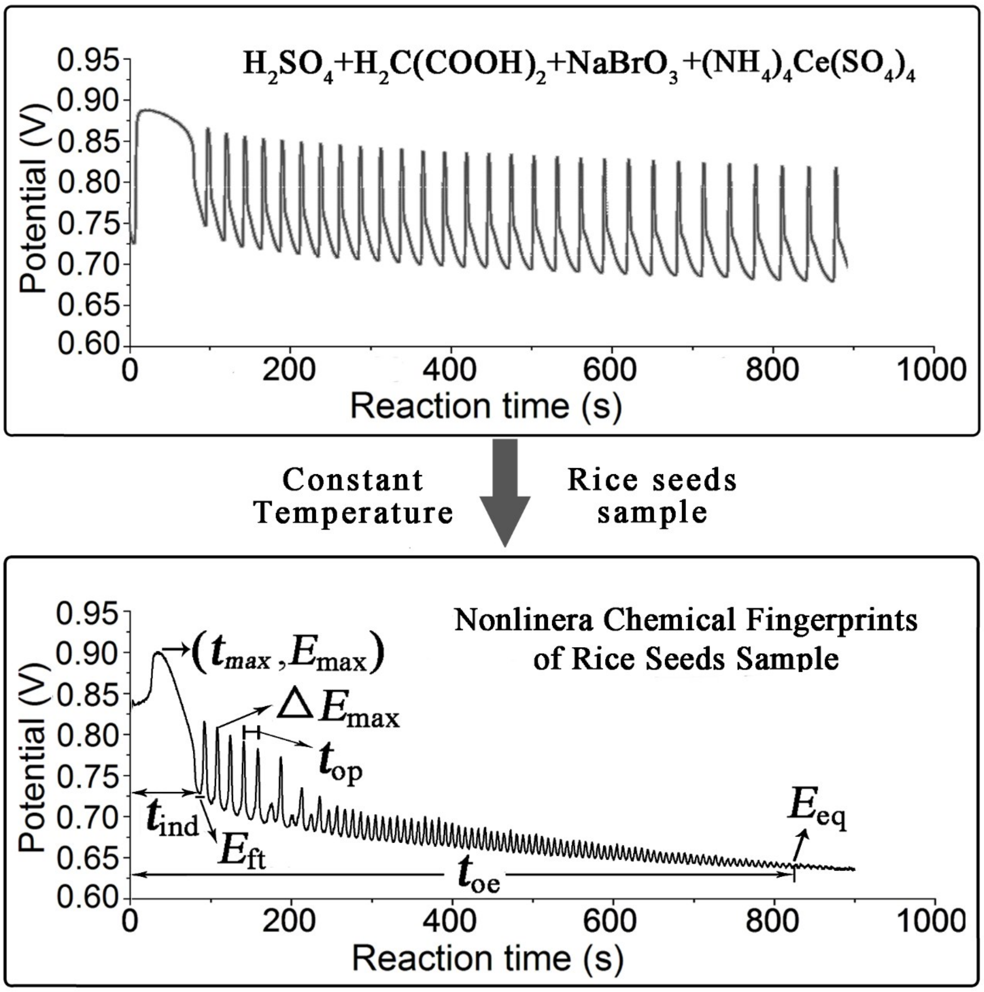

Graph abstract. B-Z oscillation reaction can be maintained for several hours without interference of foreign substances. Oscillation reaction process has changed when the sample was addition, and a characteristic fingerprint was obtained.

Study on Identification of Rice Seeds by Chemical Oscillation Fingerprints

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Abstract

A variety of nonlinear chemical oscillation fingerprint was studied which produced by interaction between rice seeds and B-Z oscillation system. Chemical constituents and its relative content in the rice seeds could be indirectly reflected by the oscillation fingerprints, and the characteristic parameters of oscillation fingerprint could be automatically extracted by program. The result showed that all rice seed samples used in the experiment could be identified accurately by both the principal component analysis and the cluster analysis, and the identification process could be efficiently completed in 10 to 20 minutes. The method had many advantages in application, such as simple operation, low cost, rapid identification and so on.

Keywords: Rice seeds, chemical oscillation fingerprint, pattern recognition, B-Z oscillation reaction

1. Introduction

Rice is an important food crop, consists mainly of starch, and one third population in the world take rice as the main food. The success of three-line hybrid rice has put the rice plant onto a new stage. Hybrid rice has obvious heterosis phenomenon, such as developed root system, large grain, strong resistance, etc. In recent decades, growing numbers of new varieties of rice were cultivated, meanwhile the market demand for high quality rice seed was also growing. Naturally, accurate identification of rice varieties had become a requirement for management. In addition, hybrid rice was heterozygous, and the genes would change in the next generation. As a result, farmers had to purchase seeds from the seed market every year. Huge benefits had led to the birth of the criminals. Fake seeds on the market further highlighted the requirement for identification. Fake seeds, which had low yield and poor quality, were mostly produced by small workshops, resulting in small economic benefits of farmland, or even no harvest, and poured into the market would cause huge economic loss for farmers. Thus, looking for methods to identify rice varieties efficiently was urgent and significant for seed quality management and safeguarding interests of farmers.

The existing identification methods of crop seeds could be broadly divided into two categories which was morphological identification and biochemical properties identification. The morphological identification method discriminated crop varieties by directly observed the appearance of seed or seedling. [1,2] The method was simple and don't need any complex instrument, however there were some factors which restricted the application. For example, the appearance of rice seeds was very similar, observation experience would inevitably restricts the accuracy of identification, and it taken a lot of time to cultivated seedlings. Electrophoresis and DNA molecular markers were the two most important methods in biochemical identification. Protein electrophoresis was a representative of electrophoresis, and the method had been widely used in the identification of maize and wheat varieties.[3-5] Electrophoresis was a low cost and environment friendly method, but the sample preparation was complicated and the separation of the protein was time consuming. DNA molecular markers include AFLP,[6] RAPD,[7] SSR,[8,9] etc. The identification precision of DNA molecular markers was high, but the operation was complex, and most of the instruments used in the methods were expensive. So, each identification methods had its unique advantages, but also limitations. Therefore, it was necessary to develop different analysis methods from multiple angles to complement each other, enabling the identification of complex biological samples easier, faster and more accurate.

In 1958, the Russian chemist Belousov and Zhabotinskii first reported the chemical oscillation reaction that citric acid could be oxidized by potassium bromate under the catalysis of cerium ion, and the solution was periodically changed between yellow and colorless, what was the B-Z reaction.[10-12] In 2003, Chinese experts Li reported a new method for identifying traditional Chinese medicine based on B-Z oscillation reaction,[13] and then T. M. Zhang, Z. H. Chen et al, made a further study on the principle of chemical oscillation fingerprint.[14-16] In this paper, we identified rice varieties using chemical oscillation fingerprints, and there was no related study be found by the method. The results show that rice seed varieties could be accurately and effectively identified with advantages of simple sample pretreatment, simple operation, low cost, and rapid analysis.

2. Experimental

2.1 Reagents and chemicals

A total of 21 rice varieties were studied in the experiment. Baixiang, Sixiang and Yuxiang were traditional rice varieties and all of the other were hybrid rice. Traditional rice seeds were purchased from Guangxi Liuzhou Seed Co., and hybrid rice were purchased from Zhejiang Lixin Seed Co., Ltd. The FYongyou10 was the second generation seed of Yongyou10, which was used to simulate the low purity of the fake seeds. Similarly, the second generation seed of Wuyou1 was marked as FWuyou1. All seeds were produced in China, and moisture content was 13%.

Concentrated sulfuric acid was diluted with ultrapure water to 1.00 M. Then prepared $CH_2(COOH)_2$ solution (0.25M) and $(NH_4)_4Ce(SO_4)_4$ solution(0.08M) in 1.00 M H_2SO_4. $NaBrO_3$ solution (1.25M) was prepared in ultra-pure water. All chemicals used were analytical reagent grade without further purification, and all reagents were purchased from Kelong chemical (group) Co., Ltd. (Chengdu, China).

2.2 Apparatus

Rice seeds were crushed in Type Q-250B high-speed multi-functional grinder (Shanghai Bingdu Co., Ltd.).

Oscillation experiments were performed in MXlab chemical oscillation fingerprint instrument which was developed in our laboratory.[17] It consist of glass reactor, circulating water bath, magnetic stirrer, temperature controller and data acquisition device with USB interface. The reactor was composed of two layers of glass with a hollow interlayer, and warm water could be circulated between the interlayer and the heating device. Potential was detected by a Model 213 platinum electrode (Tianjin, China) and a satisfied copper sulfate electrode (it was made in our laboratory) as reference electrode. Potentials (E) of the electrode as a function of time (t) were recorded to obtain a kinetic curve (E-t) of the oscillation reaction by the instrument. The experiments could also be performed by conventional reactor and devices, such as electrochemical workstation.

2.3 Sample Pre-treatment

At first, 50.0 g of rice seeds were put into a multi-functional grinder to be crushed for 90.0 seconds. After crushing, filter powder samples with 80 mesh strainer to get rid of large particles. Finally, store the powder samples in sealed bag.

2.4 Experimental Procedures

At first, 50.0 ml of malonic acid (0.250 M) was put into reactor and the temperature of water bath was adjusted to 45.0 °C. Then, the magnetic rotor was continuously stirred at 480 rpm for 5 min to ensure that the temperature of the reactant reached equilibrium. Later, 1.50 g rice seed samples were added into the reactor, and 1.00 ml $NaBrO_3$ (1.25 M) solution and 1.00 ml ammonium ceric sulfate solution (0.0800 M) were added into reactor rapidly after the samples were mixed well. At last, data acquisition was started, and potential changes were recorded with 0.1 s of sampling interval until the potential oscillation disappeared.

3 Results and Discussion

3.1 Principle of chemical oscillation fingerprints of the rice seeds

Identification of rice varieties by nonlinear chemical oscillation fingerprints was based on B-Z oscillation reaction. The reaction mechanism was explained by many kinetic models by many scholars, the widely accepted one was FKN model proposed by Field, Koros and Noyes which had been used to explain and describe many properties of B-Z oscillation chemical reactions.[18,19] There were more than twenty elementary reaction steps in the model, and an arbitrary element reaction which was disturbed by external chemical substances can cause the change of oscillation reaction process. So, the oscillation system was very sensitive to external chemical substances.

$(NH_4)_4Ce(SO_4)_4$ was catalyst in the reaction, and cerium ion periodically varying between Ce^{3+} and Ce^{4+} lead to potential changes periodically in B-Z oscillation reaction. Then, a platinum electrode was employed to detect the potential change in the reaction, and the potential (E) used as a function of time (t) was recorded by computer. The active constituents of rice seeds could directly or potentially influenced processes of oscillation reaction, and further reflect in characteristics of the chemical oscillation fingerprints. Potential amplitude would be significantly inhibited and equilibrium time would be shortened when rice seed sample was added into the oscillation system. The shape and characteristic of oscillation fingerprints were markedly different due to the different redox substances and its relative contents in different rice seeds. It was difficult for counterfeiters to evade detection through the control of one or several chemical component, because the characteristics of the oscillation fingerprint was obtained by the comprehensive effect of all the complex chemical substances in the rice seeds. In this way, a particular chemical oscillation fingerprint (E - t) of rice seed had be obtained.

Chemical oscillation fingerprint contains a wealth of information, and its characteristic could be described by many parameters. For exmple, oscillation not started immediately when all reagents were added to the reactor but started a moment later. Induction time (t_{ind}) was used to describe this interval. Meanwhile, the potential reached a maximum in the interval, and the maximum point be marked as maximum potential (E_{max}). Then, the potential falled to the first trough (E_{ft}) and oscillating periodically. In the same way, each potential of the peaks and troughs was extracted as a parameter. The oscillation reaction was considered to reach equilibrium when the amplitude of the potential was less than 0.005V. The time required for reach the equilibrium was called oscillation life (t_{oe}). The characteristic parameters of oscillation fingerprints could be automatically extracted by program. Principle of the program was simple, and it was similar to finding the maximum and minimum values in a certain range. The program could find each inflection point by comparing the surrounding data, and it was used to extract the highest potential and lowest potential in each oscillation cycle. Finally, the properties of each varieties of rice seed were stored in a database. In this way, varieties of unknown samples could be identified by comparing the database, and the more parameters of rice varieties were collected, the more rice varieties can be Identified. Although, some reaction conditions, such as temperature and composition of the oscillation system can affect the characteristics of the oscillation fingerprints, and rice seeds could showed their unique characteristics in unified conditions. The acquisition of chemical oscillation fingerprint was shown in Figure 1.

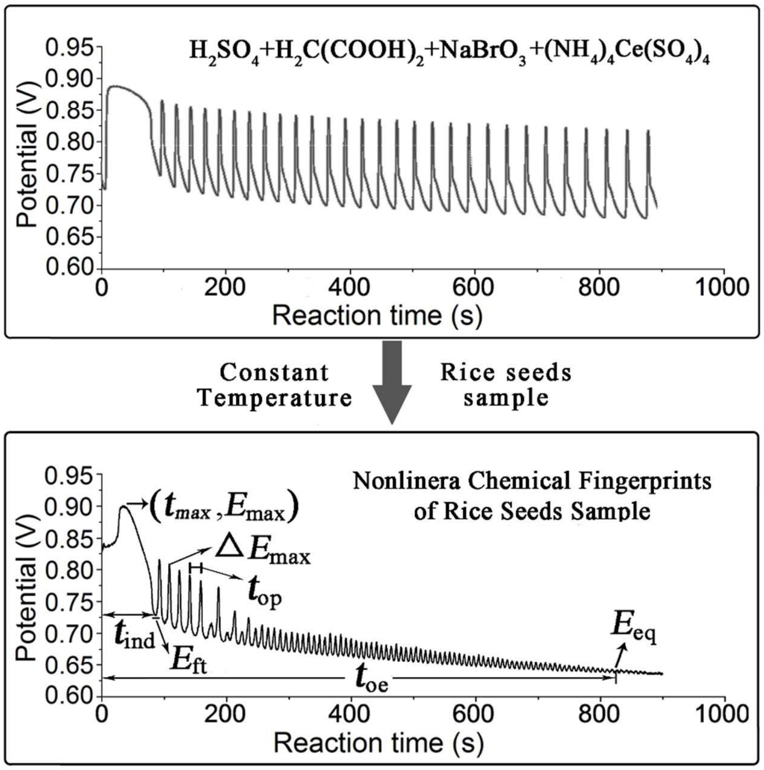

Figure 1. B-Z oscillation reaction can be maintained for several hours without interference of foreign substances. Oscillation reaction process has changed when the sample was addition, and a characteristic fingerprint was obtained.

3.2 Effect of temperature on reaction time

We explored a suitable temperature what allowed the identification to complete efficiently. All the reagent dosage and reaction conditions except the variable were performed according to the Experimental Procedures. In the preliminary test, an appropriate increase in temperature helped to speed up the reaction process, but high temperature also had a negative effect on the reproducibility of the oscillation reaction. So, we studied the chemical oscillation fingerprints of different rice seeds between 30°C and 55°C by single factor experiment. In this range, the fingerprint could clearly expressed the unique characteristics, and the reproducibility was well. According to the experimental procedure, the experimental results were shown in the Figure 2.

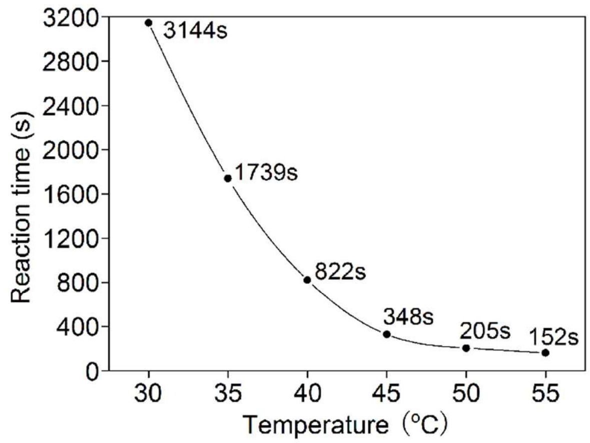

Figure 2. Temperature can significantly affect the reaction rate, and an appropriate increase of temperature can reduce the identification time.

As shown in Figure 2, like most reactions, the temperature had a great influence on the reaction rate. When the temperature was 30.0 °C, the oscillation reaction need 3144s to reach equilibrium state. Then, with the increase of temperature, the reaction time decreases sharply. When the temperature was raised to 45.0 °C, the equilibrium state can be reached within 6 minutes. The reaction time did not decrease significantly, and it taken more time to heat circulating water when we further increased the temperature higher than 45.0 °C. In summary, 45 °C is a turning point, and the identification could be accomplished quickly in this temperature.

3.3 Additional amount of samples

We studied the influences of additional amount of rice seed sample on chemical oscillation fingerprint by single factor experiments in accordance with the Experimental Procedures, and the rice seeds used in the experiments were the Yongyou10 which had a strong inhibition to make the reaction reached equilibrium state in short time. The experimental results were shown in the Figure 3.

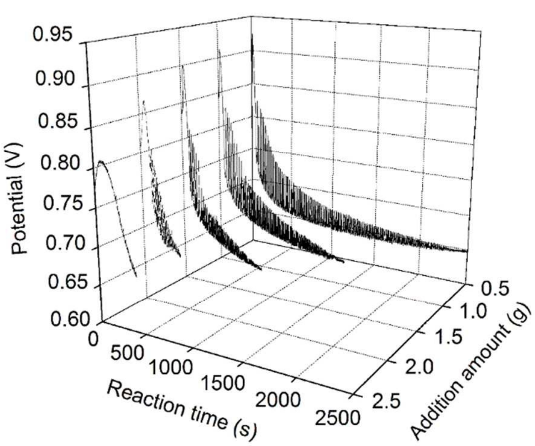

Figure 3. The addition amount of the sample significantly affected the characteristics of chemical oscillation fingerprint.

As was clearly shown in the figure, when the additional amount of sample was 0.5g, the equilibrium time of oscillation reaction was close to 2500s. Also, we can see an obvious trend that with the increasing amount of the sample, the equilibrium time and oscillation period was shortened, and the maximum potential was decreased. When the additional amount of sample was increase to 2.50g, the oscillation was completely inhibited, and oscillation fingerprint will lose all the parameters. Taking into account the extension of the number of rice samples in the future experiments, we used 1.50g as the optimum additional amount of sample. This was a conservative addition amount which could reflected characteristics of fingerprint clearly and quickly, also reserved sufficient margin to ensure the characteristics of most rice seed samples can be reflect clearly.

3.4 The parameters of chemical oscillation fingerprints of different rice varieties

Eight parameters were extracted to describe the characteristics of the oscillating fingerprints, which were maximum potential (E_{max}), time of maximum potential (t_{max}), maximum amplitude of potential (ΔE_{max}), the potential of the first valley (E_{ft}), the potential of the last peak (E_{lp}), induction time (t_{ind}), oscillation period (τ_{op}) and oscillation life (t_{oe}). Each rice variety was measured ten times, and the mean values of parameters be list in the Table 1.

Table 1. Characteristic parameters of chemical oscillation fingerprints of different rice varieties

	t_{max}/s	E_{max}/V	ΔE_{max}/V	E_{ft}/V	E_{lp}/V	t_{ind}/s	τ_{op}/s	t_{oe}/s
Y2you2	**12.2**	**0.833**	**0.749**	0.679	0.588	74.5	17.0	496.3
Y2you689	32.3	0.868	0.771	0.715	0.611	85.9	14.5	628.5
Y2you5867	33.8	0.875	0.787	0.726	0.631	97.9	13.0	535.6
Nei5you8015	18.9	0.845	0.770	**0.679**	0.587	69.1	20.0	551.5
Chunyou84	23.0	0.858	0.783	0.709	0.604	68.6	21.1	606.1
Guang2youxiang	40.7	0.878	0.806	0.736	0.612	81.2	15.6	800.5
Shen2you5814	27.0	0.865	0.784	0.725	0.635	78.1	17.7	550.3
Tianyou998	16.5	0.844	0.774	0.691	**0.551**	**56.1**	17.9	907.4
Tianyouhuazhan	25.6	0.867	0.794	0.721	0.596	68.9	19.8	**1026.3**
Wuyou1	30.9	0.878	0.798	0.738	0.641	77.4	12.8	731.4
Wuyou308	**121.6**	0.838	0.787	0.745	0.631	**153.8**	10.0	691.9
Yang2you6	20.3	0.864	0.785	0.708	0.590	69.9	24.5	845.3
Yongyou9	68.7	0.876	**0.812**	**0.755**	0.678	107.4	11.8	486.1
Yongyou10	58.5	0.874	0.790	0.731	**0.679**	113.5	**11.2**	**373.5**
Yongyou15	31.2	**0.885**	0.789	0.735	0.665	91.1	18.2	631.4
Zhu2you2	20.3	0.862	0.786	0.718	0.588	74.7	15.3	922.4
Baixiang	22.2	0.866	0.793	0.709	0.590	68.5	**22.2**	709.6
Sixiang	20.3	0.855	0.780	0.704	0.587	63.2	19.1	663.1
Yuxiang	22.2	0.870	0.793	0.708	0.584	62.3	20.1	683.2
FWuyou1	25.9	0.822	0.761	0.709	0.585	65.0	14.4	629.2
FYongyou10	38.5	0.836	0.759	0.680	0.613	83.1	16.2	361.4

As was clearly shown in the table 1, every rice seeds had unique and different parameters, and all parameters shown significant differences between different kinds of rice seeds. For example, Tianyouhuazhan rice had the longest oscillation equilibrium time, which was 1026.3s, while the Yongyou10 with strong inhibitory effect made the potential oscillation reached equilibrium state in just 373.5s. Moreover, Tianyou998 had the shortest inductive time, which was 56.1s, while the inductive time of Wuyou308 was up to 153.8s, almost three times the former. The obviously differences not only in time, but also in potential. For example, the maximum potential amplitude of Yongyou9 was 0.812V while the maximum potential amplitude of Y2you2 was only 0.749V.

3.5 Reproducibility of chemical oscillation fingerprints

The chemical oscillation fingerprint of Yongyou10 was measured 10 times under the optimal reaction conditions to investigate the reproducibility.[20] All parameters were extracted by program, and the results of experiment be shown in Figure 4 and Table 2.

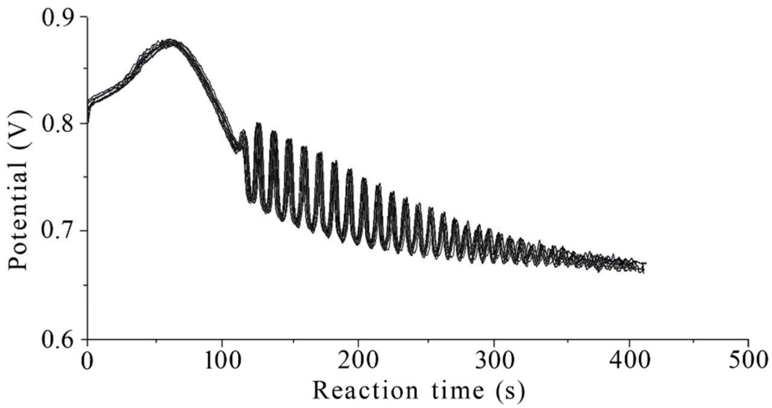

Figure 4. The fingerprint of Yongyou10 was measured ten times in the same condition.

Table 2. Characteristic parameter of the chemical oscillation fingerprints with the sample Yongyou10

	t_{max}/s	E_{max}/V	ΔE_{max}/V	E_{ft}/V	E_{lp}/V	t_{ind}/s	τ_{op}/s	t_{oe}/s
Mean Value	58.0	0.875	0.790	0.731	0.680	114.1	11.1	375.7
RSD/%	3.0	0.5	0.7	0.4	0.6	1.2	1.9	2.6
Uncertainty	1.2	0.003	0.004	0.002	0.003	1.1	0.2	6.9

The uncertainty of measurement was calculated by Bessel formula, and confidence probability was 95% in t distribution. From the experiment data, the chemical oscillation fingerprints overlapped well, and the maximum RSDs of characteristic parameters were not more than 3.0 %, which showed that the chemical oscillation fingerprint had high reliability and good reproducibility.

3.6 Identification of chemical oscillation fingerprints of rice seeds by principal component analysis (PCA)

In order to facilitate and intuitive analysis the parameters of oscillation fingerprints, we reduced the dimensions of the original parameters by principal component analysis.[21] All calculations were performed in the Matlab software. At first, raw parameters need to be standardized to eliminate the impact of different magnitude and unit, and the covariance matrix was established based on standard matrix. Later, eigenvalues and contribution of the components were obtained by calculation, and the number of principal components was determined by accumulated contribution. The result show that, the contribution of first principal component (PC1) was 53.2%, the contribution of second principal component (PC2) was 25.4%, the contribution of the third principal components (PC3) was 11.6%, and accumulated contribution of the three principal components was 90.2%. This means that the three components can include the 90.2% information of raw parameters. At last, PC1, PC2, PC3 were used as the new parameters to draw a 3D scatter diagram to show the differences in rice samples in a visual ways (Figure5).

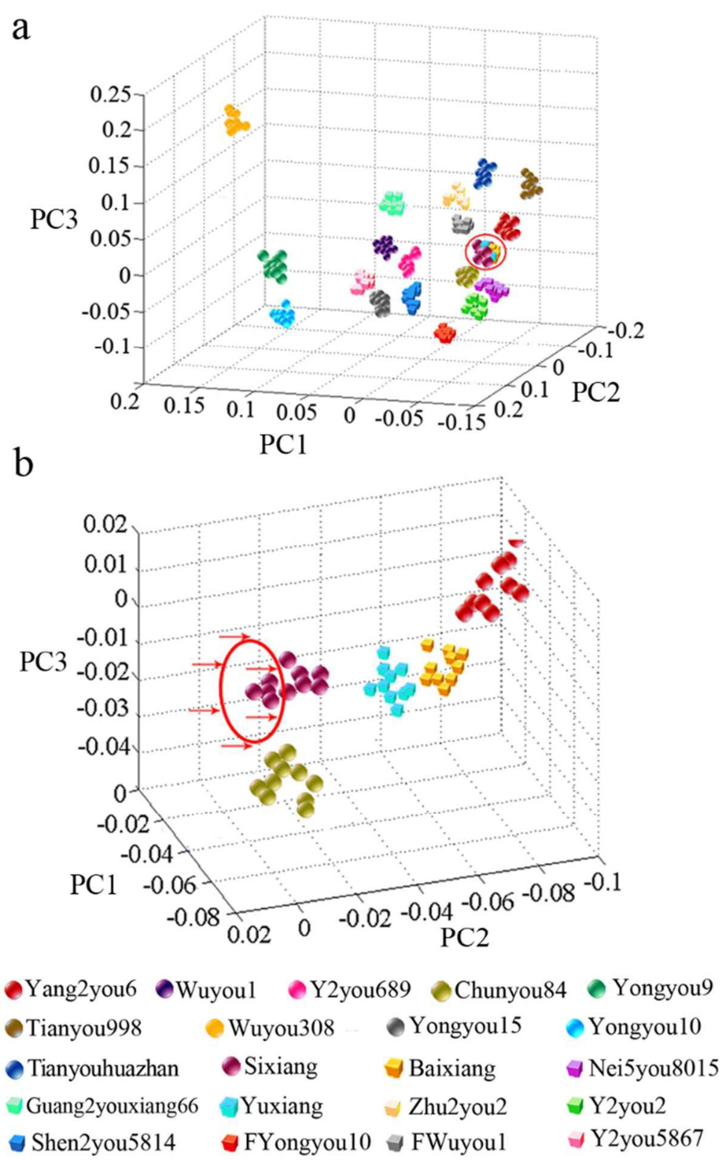

Figure 5. PCA of 210 rice seed samples

As shown in Figure 5a, the 210 samples of rice seeds were divided into different regions according to the characteristic parameters. Rice seed samples of the same variety were gathered together and keep distance with other varieties. This shows that the chemical compositions of different rice seeds were obvious differences. Two samples of fake seeds, FWuyou1 and FYongyou10, were each divided in a separate area which shows that chemical composition of fake seeds were different from any varieties used in the experiment. In the marker region, spatial location of Sixiang, Yuxiang and Baixiang could not be clearly distinguished because of the limited perspective of figure 5a. So, a partial enlargement was provided for better observation. Figure 5b provides a different perspective, and the red arrow was the observation angle of Figure 5a. From perspective of Figure 5b, Sixiang, Yuxiang and Baixiang were actually well separated. This indicated that all varieties could be separated from each other in 3D scatter diagram. In summary, PCA was a convenient and effective method for identification of rice seeds, and the results was intuitive and clear.

3.7 Identification of chemical oscillation fingerprints of rice seeds by cluster analysis (CA)

Based on eight characteristic parameters of chemical oscillation fingerprints, 210 samples of rice seeds were analysed by cluster analysis with the Matlab software. After standardization of the characters of the original data matrix, the correlation and distance coefficients were respectively computed by Euclidean distance method and Single link method. Then the cluster analysis was given as a dendrogram, and the result was shown in Figure 6.

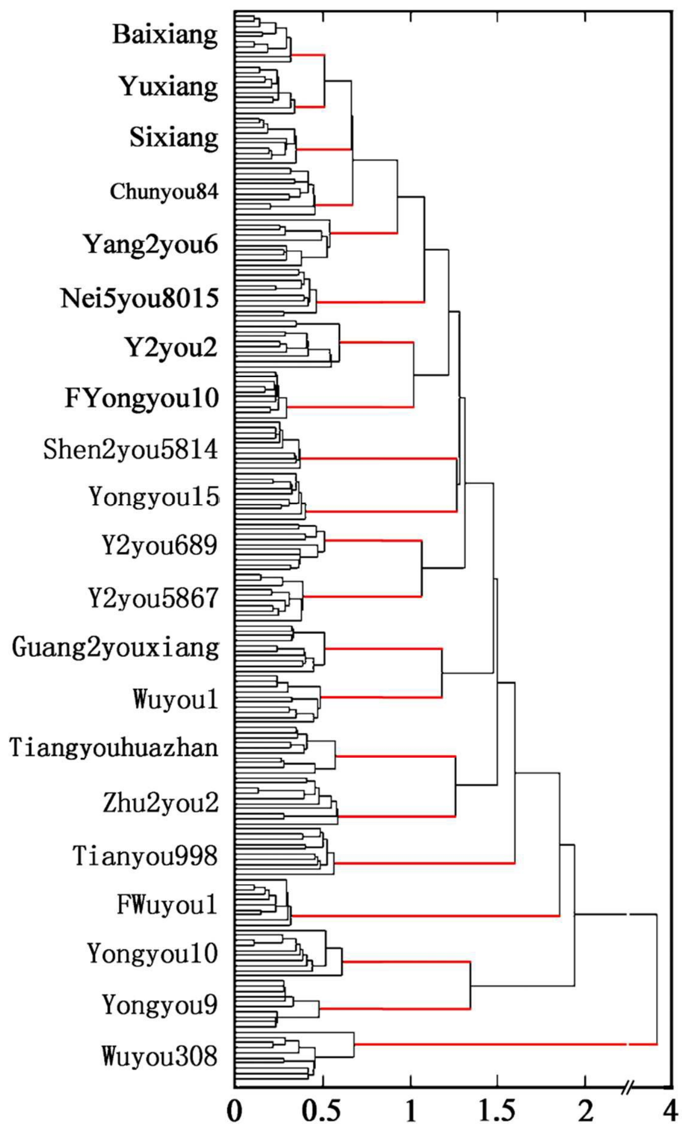

Figure 6. Dendrogram of cluster analysis of 210 rice seed samples

As shown in Figure 6, in the right side of dendrogram, all rice samples were roughly divided into two categories: Wuyou308 and the others, which means the chemical composition of Wuyou308 had great differences with the other rice varieties. From right to left, the classification gradually became detailed, and all rice samples were divided into 21 categories at red line. It's indicates that 19 varieties of rice seeds and two varieties of fake seed can be accurately distinguished in the dendrogram. Also, we can see the similarity between rice varieties from the dendrogram. For example, Baixiang were divided into same categories as Yuxiang at upper branch, which show that the chemical compositions of Baixiang and Yuxiang were very close. In summary, all rice samples could be separated according to varieties, and the result of CA consistent with the PCA.

4 Conclusions

This paper aims to identify rice seeds varieties using chemical oscillation fingerprints combine with pattern recognition. Chemical constituents and its relative content in the rice seeds can be indirectly reflected by the chemical oscillation fingerprints which produced by the interaction between rice seed samples and the B-Z oscillation system, and the rich information in the oscillation fingerprints could be extracted and represented by eight parameters. The result show that all rice seed samples used in the experiment could be accurately identified by both the CA and PCA. The biggest advantages of chemical oscillation fingerprints is the rapid identification and simple operation compared with the traditional methods. First of all, soluble solid and liquid sample can be directly added to the reactor without sample pre-treatment, and insoluble solid sample can be used after a simple and rapid grinding processing. Secondly, the interaction between samples and oscillation system is fast, and most oscillation fingerprint can be obtained within 20 minutes. Finally, parameter extraction and data analysis can be accomplished by the program, and the identification results can be displayed in a few seconds. Thus, the fingerprint is faster than the common identification method, such as electrophoresis and DNA molecular markers. Low price is also an important advantage. Although the identification precision of DNA molecular markers was higher than chemical oscillation fingerprints, the instrument price of the latter is less than 1/100 of the former. It is very helpful for promotion of the method in developing countries.

In the future work, we will improve the identification method and experimental instrument to further improve sensitivity, and enabling the identification to be completed in trace sample requirement. We are trying to apply the method in more areas, such as medical detection. In conclusion, chemical oscillation fingerprints is an effective method for the identification of rice seeds. Rapid identification, low cost, convenient operation all make the method shows a bright prospects on application.

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