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Direct evaluation of meat spoilage and the progress of aging using biosensors

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Abstract

A direct sensing method for monitoring meat quality was developed. The sensor is composed of an Ag/AgCl electrode and a platinum electrode on which putrescine oxidase or xanthine oxidase were immobilized to estimate bacterial spoilage or the progress of aging, respectively. A potential-step chronoamperometric method was applied in which the potential was stepped from 300 mV to 600 mV. A linear relationship was obtained between 5 and 60 nmol g^{-1} for putrescine (Put) and 0.05 and 1.0 μmol g^{-1} for hypoxanthine (Hx). The coefficient of variation was 0.75% for 20 nmol ml^{-1} Put solution and 2.2 for a meat sample using the putrescine sensor, and 1.09% for 0.25 μmol ml^{-1} Hx solution and 2.6% for a meat sample using the xanthine sensor. The pH requirements and substrate selectivity were suitable for the direct measurement of substrates on the surface of meat. From the results of practical experiments, the direct sensing method was indicated to be useful with some modifications for the estimation of meat quality during aging.

Keywords: Biosensors; Meat freshness; Putrescine; Xanthine

1. Introduction

Meat, especially beef, reaches an acceptable state for consumption after a long period of storage at low temperature, a storage procedure known as aging. During storage, not only aging but also bacterial spoilage can occur. Consequently, to obtain appropriately aged meat, it is desirable to monitor the progress of aging and bacterial spoilage simultaneously.

To estimate bacterial spoilage, biogenic amines, especially putrescine and cadaverine, have been confirmed to be useful chemical indicators [1–4]. Hypoxanthine, a degradation product of ATP, was confirmed to be a useful chemical indicator for estimation of the progress of aging [5,6].

Based on these findings, a meat freshness sensor composed of a 2-line flow injection analysis (FIA) system was developed [7]. This sensing system was composed of a putrescine sensor and a xanthine sensor and could be used to evaluate the progression of both aging and bacterial spoilage simultaneously. As a FIA system was employed, only liquid samples could be analyzed and thus for meat samples it was

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necessary to prepare sample solutions by homogenization and filtration. Such preparation methods entail destruction of the sample and are also time consuming.

From the viewpoint of quality assurance, it is desirable to inspect all products and thus an ideal sensing method for the food industry would be on-line and non-destructive [8]. Biosensors can be used on-line and non-destructively to analyze liquid foods such as milk, soup and beverages [9]. In these cases, the sample can be supplied either automatically or manually and tested without an extraction procedure.

For solid foods such as meat, however, the application of a biosensor is very difficult. Consequently, there have been no previous studies using biosensors for testing of solid food using on-line and non-destructive methods. To construct such a biosensor, a direct sensing method is required [10].

In a study of biosensors for clinical use, Yamauchi et al. [11] reported a pulse voltammetric method which required no agitation of the sample solution. After the serum was placed on the sensor, a potential of 0.6 V was applied for 60 s and the current–time curve was recorded. The concentration of glucose in serum was calculated from the differences in transient response curves obtained with and without substrates. In this method, the potential was applied from the off state of the potentiostat.

The major difficulty in construction of a direct sensing amperometric enzyme electrode is to determine the baseline current. With the FIA sensor, the baseline current can easily be measured, and thus the difference in current between the stabilized baseline and the peak can be calculated. However, in the direct sensing method, it is impossible to determine the baseline current because the enzymatic reaction begins immediately the sensor comes into contact with the meat surface. If the baseline current is recorded in air or buffer, there is a risk of error caused by the difference between the conditions of the air or buffer and the sample surface.

Potential-step chronoamperometry is a frequently used electrochemical technique [12], and we supposed that by application of this method the difference in electric current caused by the enzymatic reaction could be determined from the difference in current before and after the potential step.

Accordingly, we developed a direct sensing system for monitoring meat freshness and meat quality during aging. The sensor used was composed of an enzyme electrode on which putrescine oxidase or xanthine oxidase was immobilized, and the method of potential-step chronoamperometry was applied.

2. Experimental

2.1. Reagents

Putrescine oxidase (from *Micrococcus rubens*) was obtained from Amano (Nagoya, Japan). Xanthine oxidase (from buttermilk) was obtained from Sigma (St. Louis, MO). Nafion was obtained from Aldrich (Milwaukee, WI). All other chemicals were of analytical grade.

2.2. Construction of the sensing system

Fig. 1 shows a schematic diagram of the direct sensing system. Working electrodes were made from platinum disks 3.0 mm in diameter. At the end of the polished Pt disk putrescine oxidase or xanthine oxidase was immobilized by glutaraldehyde treatment. To eliminate interference from uric acid in the meat sample, a Nafion membrane was formed over the immobilized enzyme. A 5 μl aliquot of Nafion solution diluted with ethanol was dropped onto the immobilized enzyme and air-dried at room temperature. This procedure was repeated twice. A polycar-

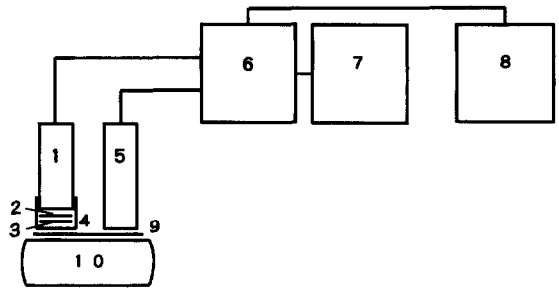

Fig. 1. Schematic diagram of the direct sensing system for evaluating meat freshness: (1) platinum electrode; (2) immobilized enzyme; (3) Nafion membrane; (4) polycarbonate membrane; (5) Ag/AgCl reference electrode; (6) potentiostat; (7) function generator; (8) recorder; (9) filter paper; (10) meat.

bonate membrane (pore size 1.0 μm, Costar, Cambridge, MA) was placed over the Nafion membrane to strengthen the surface of the enzyme electrode for contact with the sample. Measurements were made using a two-electrode system with a Ag/AgCl electrode as reference.

To decrease the fluctuations caused by the contact surface between sensor and sample, a sheet of filter paper (No. 1, Advantec-Toyo, Tokyo, Japan) was laid over the meat and the sensor was placed on the filter paper which was wetted by the exudate from the meat. When standard solutions were measured, the filter paper was moistened with the standard solution.

A typical response curve obtained by this method is shown in Fig. 2. After the output current of the sensor had stabilized at 300 mV in 0.1 M phosphate buffer (pH 7.0), the sensor was placed in contact with the filter paper on the surface of the meat. The sensor was held in place for exactly 2 min and then the potential was increased to 600 mV at 10 V^{-1} using a function generator and a potentiostat. Following the appearance of a sharp peak of the charging current, the current decreased gradually. The difference in current between that just before the potential step and that after 1 min was used to evaluate the sample. After measurement, the sensor was again immersed in buffer solution bubbled with air to clear the surfaces of the enzyme and reference electrodes until the current was stable at 300 mV.

2.3. Sample preparation

Sirloin meat from a Holstein steer obtained from a carcass stored at 0°C for 2 days after slaughtering was used. The meat was cut into 20 mm thick steaks (250–300 g), vacuum-packed in high barrier film bags (Nylon/Binding layer/L.LDPE, 210 × 420 mm, 0.07 mm thick) and stored at 5°C. Two samples of each piece of meat were used in each experiment.

2.4. Measurement of bacterial counts

Ten grams of the surface of the meat (5 cm × 5 cm) was shaved using a sterilized surgical knife and homogenized with 90 ml of sterile 0.9% saline (NaCl) solution. Decimal dilutions were spread over plates of plate count agar (Eiken, Tokyo) to determine the total aerobic viable counts. Colonies on the plate were counted after incubation for 5 days at 25°C. Duplicate experiments were performed for each specimen.

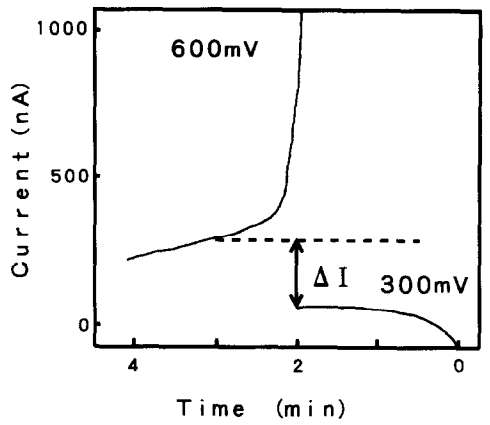

Fig. 2. Typical response curve of a sensor.

2.5. Measurement of polyamines

Ten grams of the surface of the meat was shaved, homogenized with 20 ml of 3% perchloric acid and centrifuged at 3000 *g* for 10 min and the supernatant was decanted. This procedure was repeated 3 times and the combined surpernatant was adjusted to pH 6.8. The precipitate formed was removed by centrifugation and the supernatant was made up to 50 ml with distilled water. Each sample solution was purified and concentrated for analysis by liquid chromatography (LC) [13,14], briefly, by passing the solution through a cation exchange resin (Dowex 50W) column, mainly to eliminate amino acids. The polyamine fractions were dried in a rotary evaporator and the residues were redissolved in a small amount of 0.2 M perchloric acid. Putrescine (Put), cadaverine (Cad), spermidine (Spd) and spermine (Spn) were determined by LC on a reversed-phase Shimpack CLC-ODS (Shimadzu, Kyoto) column (6.0 × 150 cm) equipped with a fluorometric detector as reported by Gamou and Fujita [15].

2.6. Measurement of ATP and related compounds

ATP, ADP, AMP, IMP, inosine (HxR), hypoxanthine (Hx) and xanthine (X) were determined accord-

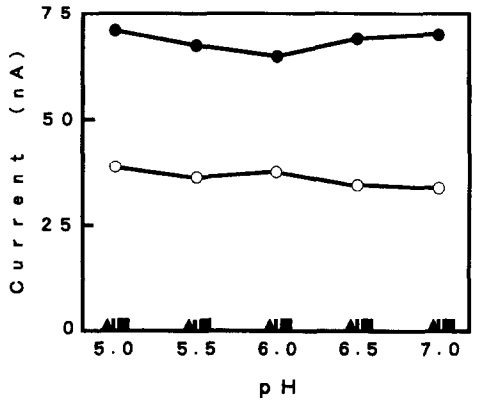

Fig. 3. Response of the putrescine sensor at various pH values: ●, putrescine; ○, cadaverine; ■, spermidine; ▲, spermine. The substrate solutions were prepared using 0.1 M sodium phosphate buffer.

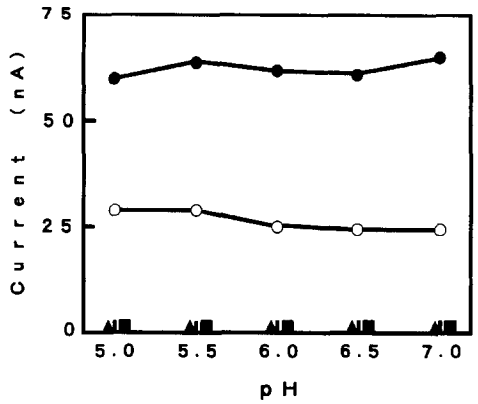

Fig. 4. Response of the xanthine sensor at various pH values: ●, hypoxanthine; ○, xanthine; ■, IMP; ▲, inosine. The substrate solutions were prepared using 0.1 M sodium phosphate buffer.

ing to the method of Yoshiura et al. [16] by LC on a Shimpack CLC-ODS column (6.0 × 150 cm) with a UV detector. Ten grams of the inner part of each meat sample was homogenized with 20 ml of 3% perchloric acid and centrifuged at 3000 *g* for 10 min. The sample solution was prepared by the same procedure used for polyamine determination and was made up to 50 ml.

3. Results and discussion

3.1. Influence of pH on the substrate selectivity

Since the pH of meat ranges between 5.0 and 7.0, it is important to study the influence of pH on the substrate specificity. Figs. 3 and 4 show the dependence of the responses of the putrescine and xanthine sensors on the pH of standard solutions. Neither sensor showed a pH-dependent response within the measured range. For the putrescine sensor, Put was assigned a relative specific activity of 100%, so Cad was about 50% while Spn and Spd showed no response.

For the xanthine sensor, Hx was assigned a relative specific activity of 100%, so X was about 50% while HxR and IMP showed no response. From these results this direct sensing method was confirmed to be applicable to the monitoring of aging and spoilage of meat samples.

3.2. Linearity of the sensor response

Figs. 5 and 6 show calibration graphs for the sensors, obtained by the potential-step method and the constant voltage method at 600 mV with aeration in buffer. By the potential-step method, a linear relationship was obtained with the putrescine sensor for Put solutions between 5 and 60 nmol ml^{-1} and using the xanthine sensor for Hx solutions between 0.05 and 1.0 μmol ml^{-1}. The difference in dynamic range between the putrescine and xanthine sensors is

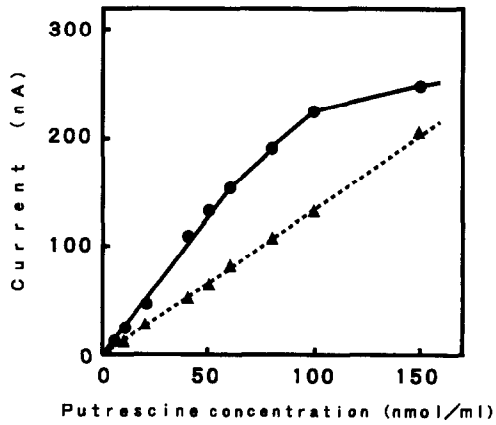

Fig. 5. Calibration graphs for the putrescine sensor: ●, potential-step method; ▲, constant voltage method at 600 mV, with aeration. The reactions were carried out at pH 7.0 (0.1 M sodium phosphate buffer).

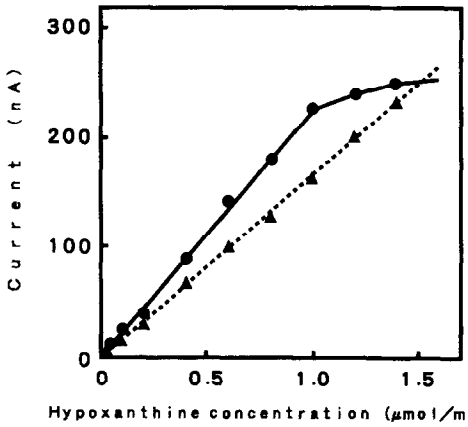

Fig. 6. Calibration graphs for the xanthine sensor. ●, potential-step method; ▲, constant voltage method at 600 mV, with aeration. The reactions were carried out at pH 7.0 (0.1 M sodium phosphate buffer).

due to the difference in the rate of diffusion of substrate and oxygen through the Nafion membrane. The diffusion rate was regulated by changing the concentration of Nafion in ethanol when preparing the Nafion membrane at the tip of the platinum electrode. Nafion concentrations were optimized at 0.5% for the putrescine sensor and 1.0% for xanthine sensor according to the results of a preliminary study (data not shown).

By using the constant voltage method with aeration, the sensor was immersed in the substrate solution, and a higher dynamic range was obtained in comparison with the potential-step method. For the putrescine sensor a linear relationship was obtained between 2 and 400 nmol ml^{-1} and for the xanthine sensor between 0.02 and 2.0 μmol ml^{-1}. In the direct sensing method, the diffusion of oxygen and substrate into the tip of the electrode were supposed to limit the enzymatic reaction rate. Thus, the dynamic range of the potential-step method was less than that of the constant voltage method.

3.3. Reproducibility of the measurements

The reproducibility of the measurements was investigated. The coefficients of variation (C.V.) using standard solutions of 20 nmol ml^{-1} Put and 0.25 μmol ml^{-1} Hx were 0.75 and 1.09%, respectively. The C.V. values on the surface of meat were 2.2% with the putrescine sensor and 2.6% with the xanthine sensor.

3.4. Stability of the sensors

To determine the stability of the sensors during storage, they were stored dry at 2°C and measurements obtained in triplicate once a week for 4 weeks. As shown in Fig. 7, the activity of the putrescine sensor showed a small decrease over this time to 84% of its original activity. The activity of the xanthine sensor, however, decreased to 16% after 3 weeks and to 0% after 4 weeks. Thus, the xanthine sensor should be used within 2 weeks of preparation.

The major problem with the sensors was the durability of the Nafion membrane. Although the Nafion membrane was protected by the polycarbonate membrane, continuous use caused it to break down in 4 out of 29 sensors; the Nafion membranes of these 4 sensors broke down after 8 to 31 times (mean ± S.D.: 20.2 ± 8.3) use. This phenomenon was indicated by the appearance of a large current caused by diffusion of uric acid from in meat. Therefore, for practical applications, methods of strengthening the Nafion membrane should be investigated.

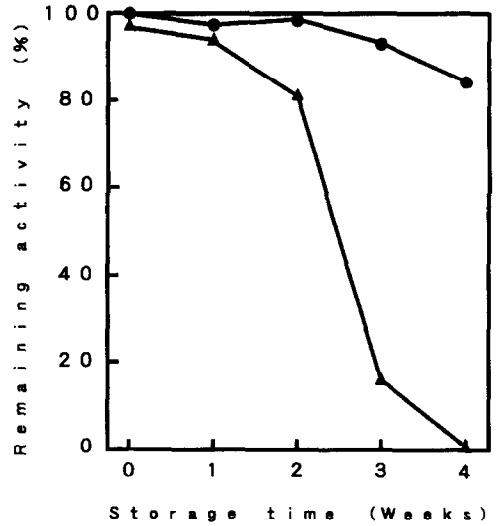

Fig. 7. Storage stability of the sensors at 2°C. ●, Putrescine sensor; ▲, xanthine sensor.

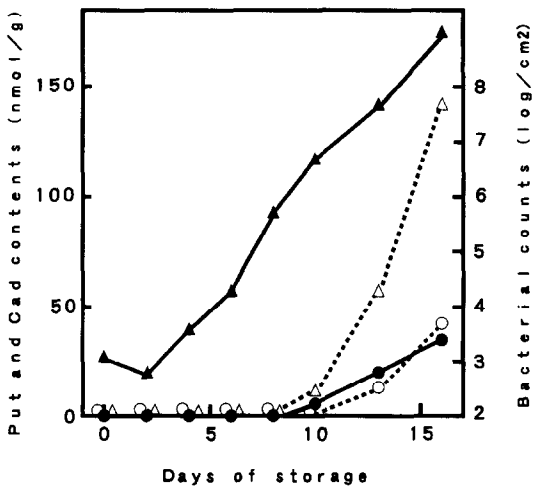

Fig. 8. Changes in putrescine and cadaverine contents measured by the putrescine sensor and by LC, and bacterial counts: ●, sensor; ○, putrescine; △, cadaverine by LC; ▲, bacterial counts.

3.5. Application of the direct sensing system to meat

To assess the utility of the direct sensing system, sirloin meat was stored at 5°C and analyzed by using the putrescine and xanthine sensors at appropriate intervals. Polyamines and ATP-related compounds were also measured by LC for comparison with the sensor-derived values.

Fig. 8 shows the changes in Put and Cad contents as measured by both the putrescine sensor and LC, and the changes in the bacterial counts. As the putrescine sensor has activity to both Put and Cad, the values determined by the sensor were expressed as Put content using the calibration graph obtained for Put solutions. Cad was first detected by LC at a concentration of 8 nmol g^{-1} after 10 days of storage, which was before the stage of initial spoilage, and increased to 141 nmol g^{-1} after 16 days. Put was first detected after 13 days, when the concentration was 12 nmol g^{-1}, increasing to 42 nmol g^{-1} after 16 days. According to the putrescine sensor, Put and Cad were first detected after 10 days when the concentration was 5 nmol g^{-1}, increasing to 36 nmol g^{-1} after 16 days. The concentrations of Spd and Spn were unchanged during storage at about 10 and 130 nmol g^{-1}, respectively.

As the sensor was half as sensitive to Cad as to Put, the sensor values should correspond to the concentration of Put + 1/2 Cad. However, these sensor values did not agree with the calculated values from the results of LC. This was presumably because the sensor measured the exudate on the surface of the meat and the Put and Cad content were much more effectively extracted by perchloric acid.

Since bacterial count is a fundamental index of meat spoilage, the sensor response should be related to bacterial count. As a bacterial count of 10^7 cells g^{-1} in meat is regarded as unacceptable [17], detection of the order of 10^6 cells g^{-1} is important as this is achieved just before the meat reaches the unacceptable stage.

In this study, samples were evaluated as fresh until 10 days old at which time bacterial counts were under 10^7 cells g^{-1} and no putrid odor could be perceived. After 13 days, the bacterial count reached the order of 10^7 cells g^{-1} and samples gave off a faint putrid odor. These samples were in the initial stage of spoilage and would be regarded as unacceptable. After 16 days, the meat gave off an obviously putrid odor and the bacterial count was in the order of 10^8 cells g^{-1}.

As mentioned above, the putrescine sensor showed a response at 10 days which was just before the unacceptable stage. After 10 days, the sensor response increased in proportion with bacterial count. Consequently, the extent of bacterial spoilage could be evaluated by the putrescine sensor. However, the sensor response should be further correlated with bacterial spoilage in further studies.

Glucose, ammmonia, pH and some other components have been proposed as chemical indices for meat spoilage, but most of these change clearly just after the meat has reached a stage of apparent putrefaction. On the other hand, Put and Cad could not be detected while meat is still acceptable, and began to be detected around the time of the initial occurrence of spoilage. Therefore, the estimation of bacterial spoilage by determination of Put and Cad is more sensitive than by other chemical indices.

Fig. 9 shows the changes in the Hx and X contents as measured by the xanthine sensor and LC. In the same way as the putrescine sensor, the xanthine sensor has activity to both Hx and X, and the values determined using the sensor were expressed as Hx

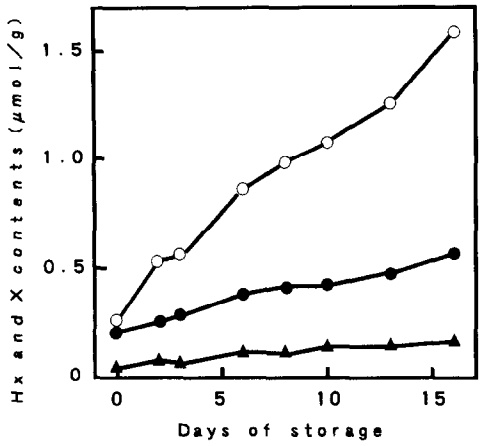

Fig. 9. Changes in hypotaxine and xanthine contents measured by the xanthine sensor and LC: ●, sensor; ○, hypoxanthine by LC; ▲, xanthine by LC.

content using a calibration graph derived from Hx solutions. As the experiment began 3 days after slaughter, ATP, ADP and AMP were not detected by LC. Other compounds showed normal changes, i.e., a decrease in IMP and increases in HxR, Hx and X. As shown in Fig. 9, Hx showed a linear increase from 0.34 μmol g^{-1} at 0 days to 1.68 μmol g^{-1} at 16 days and X also showed a slight increase during storage from 0.03 to 0.16 μmol g^{-1}. Therefore, both the Hx content and the total amount of Hx and X reflect the progress of aging. The xanthine sensor showed a linear increase in Hx and X content from 0.21 to 0.56 μmol g^{-1} but the values measured by the sensor were lower than those determined by LC; there was no correlation between the values obtained with the sensor and by LC. It was supposed that the sensor measured the exudate in the surface of the meat and LC measured the perchloric acid extract from the inner portion of meat.

After slaughter, ATP in muscle is degraded by endogenous enzymes, and Hx and X accumulate with the passage of time. The changes in Hx and X content show a high correlation with the changes in tenderness of meat which is the most apparent change caused by aging. Hence, the total amount of Hx and X has been proposed as a valuable index of the progress of aging.

Thus, to utilize the direct sensing xanthine sensor for quality control of beef aging, further studies should be performed to clarify the correlation between the sensor response and the fundamental changes occurring during the progress of aging such as tenderness and taste.

The direct sensing method measures the concentrations of substrates in the exudate on the surface of meat. This exudate contains proteins, peptides, amino acids, organic acids, purines, B vitamins and various salts [18], and it is possible to obtain useful information pertaining to quality control from the changes in concentrations of components of the exudate. The concentrations of Put, Cad, Hx and X as measured by the sensor are examples of such an approach. Therefore, further investigations should be performed to analyze the changes in other components using the potential-step biosensor.

4. Conclusions

A direct sensing method for monitoring bacterial spoilage and the progress of aging in meat was developed and applied to monitor meat quality during storage. Direct sensing was made possible by adapting potential-step chronoamperometry. The sensor could measure Put and Cad content, and Hx and X content in the exudate on the surface of meat samples. Bacterial spoilage and the progress of aging were determined from the responses of the putrescine and the xanthine sensors, respectively. From the results obtained in a practical experiment, the direct sensing method was indicated to be useful with modifications for the convenient and non-destructive evaluation of meat quality.

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