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Monitoring of ATP and viable cells on meat surface by UV–Vis reflectance spectrum analysis

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ABSTRACT

Cleanliness monitoring at slaughterhouses depend on traditional methods, *e.g.*, visual inspection or swabbing. The visual inspection is not always accurate. Swabbing requires skilled workers and further plate count or ATP bioluminescence technique. To solve these problems, a rapid technique based on non-destructive UV–Vis reflectance was developed to monitor the ATP and viable cells. Samples were lean part of pork loin. The samples stored at 15 °C were analyzed at 0, 24, 48, 72, 84 and 96 h for ATP, plate count and UV–Vis reflectance. The reflectance spectra were measured from 240 to 540 nm at 20 °C, and then the area of $40 \times 40\ mm^2$ of the sample surface was swabbed for the determination of plate count and ATP amount. The plate count on the sample surface increased from the initial count of 29 to 3.2×10^7 CFU/cm^2 after 84 h. The ATP amount also increased with time from the initial amount of 9.2×10^{-15} to 2.8×10^{-10} mol/cm^2 after 84 h. The linear relationship was observed between the ATP amount and plate count with the determination coefficient of 0.95. The 2nd derivative of raw spectra gave a high correlation for the first 48 h with both ATP amount and viable cell count showing the determination coefficient of 0.89 and 0.83, respectively at 318 nm. The results strongly suggested that the UV–Vis reflectance spectrum analysis could be used as the real-time monitoring of ATP and/or plate count on meat surface with the optimal wavelength.

1. Introduction

Muscle foods that include both meat and poultry are an integral part of the human diet and have been so for several thousand years. However, within the past two decades public concern, as well as awareness, has been raised due to high profile food safety issues such as the BSE and foot and mouth epidemics (Fox, 2001; Pickrell and Enserink, 2001). These outbreaks, along with concerns over specific pathogenic bacteria within meats have illustrated the requirement for a rapid and accurate detection system for microbial spoilage of meats within what is a large-scale production industry whose turn over is billions of £ and $ per annum (Ellis and Goodacre, 2001).

The major role of microorganisms in the spoilage of food and the role of food as a vector for the transmission of microbes responsible for food-borne disease are well recognized. At a slaughterhouse of poultry, pork and beef, monitoring of cleanliness depends mainly on traditional methods of visual inspection, swabbing and subsequent viable cell count or ATP bioluminescence technique (Hawronskyj and Holah, 1997). This is especially important for microbial hazards associated with food process. In the case of poultry, pork and beef processing, verification of the efficacy of preventive measures to reduce or eliminate microbial hazards may be achieved by routine carcass analysis using cultural method, i.e., classical Standard Plate Count (Bautista et al., 1997). However, the development of more rapid methods on a 'real time basis' for microbiological quality control has been in the interest of scientists ever since routine microbiological analysis was applied to foods. Rapid detection methods based on the detection of whole cells or their metabolites can be divided into two main classes: direct methods are based on the detection of cells with or without incubation and indirect methods are based on the measurement of metabolic products or other changes caused by the cell growth (Vanne et al., 1996).

Although rapid detection methods have been under development, conventional methods for microbial monitoring are used on the job site of slaughterhouse. However, such methods usually

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require operator's skill, long analysis time and high expenses. Moreover, visual inspection is not always accurate, the swabbing requires skilled worker and further plate count analysis which usually requires 24–48 h. The conventional microbiological approach to food sampling has changed over the last half century and it has been estimated that there are currently in excess of 40 methods to measure and detect bacterial spoilage in meats (Jay, 2005; Nychas et al., 1988). The development of rapid microbiological test procedures over the last two decades can be divided into two main groups: enumeration and presence–absence tests. Several commercial presence–absence (P-A) test kits area available and were evaluated over a 6-month period in 1990 by using the Ontario Ministry of the Environment P-A test for comparison by Clark and El-Shaarawi (1993). Current rapid enumeration methods are generally based on microscopy, ATP bioluminescence or the measurement of electrical phenomena (Ellis and Goodacre, 2001).

The use of ATP bioluminescence assay is a logical approach and relies on the fact that all living cells contain adenosine 5'-triphosphate (ATP), which is a universal energy donor for metabolism (Bautista et al., 1997). Detection of the high-energy molecule adenosine triphosphate (ATP) extracted from cells is a widely used indirect assay method. The ATP amount is measured as the light energy released by the luciferin–luciferase system in the presence of magnesium ions (Stanley, 1989). The assay is rapid, only a few seconds in hygiene monitoring applications and less than an hour of most other samples. Previously, it was thought that this technology has limitations – because of the fact that ATP is present in all viable cells. Therefore, intrinsic ATP originating from the target cells must be removed enzymatically before the assay (Vanne et al., 1996). Siragusa et al. (1996) stated that the major challenge in using microbial ATP as a means of determining total microbial populations in food samples is the separation of nonmicrobial ATP from microbial ATP. The basis of their described Rapid-microbial ATP assay was the use of a filtration device in which somatic ATP was extracted: then within the same device, extraction of bacterial ATP was followed by its quantification.

In the case of microscopic methods sophisticated techniques have been developed where microorganisms are stained with fluorescent dyes and viewed with an epifluorescent microscope. ATP bioluminescence acts by measuring ATP levels in bacterial cells in culture in order to calculate the number of cells present in that culture (Champiat et al., 2001; de Boer and Beumer, 1999; D'Souza, 2001; Siragusa et al., 1996). The problem with this method is that ATP is the primary energy source of all living cells and the food samples themselves will also contain large amounts of this chemical which have to be destroyed before microbial ATP can be measured. Consequently, the measurement of ATP bioluminescence is probably the best suited to detection of contaminated surfaces on equipment and machinery associated with food production and preparation (Ellis and Goodacre, 2001).

In the case that the viable cells should only be detected, the above-mentioned limitation of ATP bioluminescence technology and drawback of microscopic method have to be taken into account. However, the total amount of ATP originating from both meat and viable cell has sufficient importance in the cleanliness evaluation, because the ATP of meat origin acts as a nutrient source for the bacteria leading to bacterial spoilage.

Due to the advantages of nondestructive, free of chemical preparation and fast inspection speed, spectroscopy has been studied extensively for determining properties of agricultural products, but less for meat products as compared to plant materials (Chan et al., 2002).According to the literature, VIS/NIRS technology has been used in pork to determine intramuscular fat (Hoving-Bolink et al., 2005; Savenije et al., 2006), fatty acid composition (Fernandez-Cabanas et al., 2007; Gonzalez-Martin et al., 2003, 2005), color (Cozzoliono et al., 2003), water-holding capacity (Brondum et al., 2000), presence of RN^- genetic allele (Josell et al., 2000), and Doroc and Iberian porl neural network classification (del Moral et al., 2009), but it has not been applied for the direct qualitative classification of meats of varied quality and price (del Moral et al., 2009). Moreover, only a few reports are available for determination of quality of food products by using reflectance data. From these current conditions, the objective of this study was to develop a real-time detection method for monitoring of ATP and viable cells on meat surface by using reflectance spectra that could be used for sanitation management.

2. Materials and methods

2.1. Meat samples

The lean part of pork loin samples sliced in 5-mm thick was obtained from a retailer. It was slaughtered 3 days ago and kept in the marketing conditions at the retailer shop. A total of 24-sliced samples were cut into pieces of about 6×6 cm^2, and were individually placed in sterilized Petri dishes.

2.2. Experimental setup

The samples were separated into six groups with four samples of each and were stored in a constant temperature chamber at 15 °C. The storage temperature was selected as the highest temperature in a working room of a slaughterhouse, where the temperature is usually controlled from 10 to 15 °C in consideration of worker's health, according to our conversation with the slaughterhouse management. Measurements were conducted after 0, 24, 48, 72, 84 and 96 h of storage. The each value shown is a mean of four pieces. The experiment was repeated thrice to validate the results. Similar results were obtained in all the repeated experiments. Here, for simplicity, results of only one experiment are shown.

2.3. UV–Vis reflectance spectrum

A dual beam spectrometer (UV-3600, Shimadzu Co., Kyoto, Japan) equipped with an integrating sphere setup was used for recording reflectance spectrum from a surface of meat sample (9×20 mm^2). Measured range of wave length was 240–1200 nm with the resolution of 2 nm; however, the results from 240 to 540 nm are only shown in the Section 3. In order to confirm the maximum absorption wavelength of ATP, the transmittance of serial dilutions of ATP standard solution (LL-100-1, TOYO B-Net Co., Tokyo, Japan) was obtained with 10 mm quartz cells.

2.4. Spectral data pre-treatment

Spectral data are often pre-processed to reduce undesirable systemtic noise, such as baseline variation, light scattering, path length differences and so on, and enhance the contribution of the chemical composition (Tigabu and Oden, 2002). In this study, two types of pre-processing were employed: Savitzky–Golay 1st and 2nd derivative. In our case, the possible sources of systematic variation could be due to the path length slight difference arising from the positioning of individual meat samples with slight different sizes during scanning.

2.5. Sampling protocol and microbiological analysis

2.5.1. Sampling protocol

Sampling of materials on pork meat surface (40×40 mm^2) covering the area for spectroscopic measurement was carried out using a swab technique. To ensure adequate sampling, the sample

was swabbed in a horizontal pattern and again in a vertical pattern being rotated between the index finger and the thumb in a back and forth motion according to Bautista et al. (1997). The end of cotton bud used for swabbing was cut into 9 ml of sterilized water and then the swab sample was stirred well for the further examination for plate count and ATP determination.

2.5.2. Plate count

Serial dilutions of the swab sample were prepared from the phosphate buffer solution in which the swab was immersed and 1 ml of the dilution was dispensed onto Petrifilms™ (AC plate, Sumitomo 3M Ltd., Tokyo, Japan) for total aerobic counts. The Petrifilms™ were incubated for 48 h at 35 °C.

2.5.3. ATP bioluminescence assay

One hundred microliters of the swab sample (phosphate buffer solution in which the swab was immersed) was injected into a fresh cuvette placed in a luminometer (Luminescenser MCA, Atto Corporation, Tokyo, Japan), and then, 100 µl of Extractant (LL-100-2, Toyo B-Net Co. Ltd., Tokyo, Japan) was added into it. After 10 s, 100 µl of Luciferin–luciferase complex (LL-100-1, Toyo B-Net Co. Ltd., Tokyo, Japan) was added, and the light output was measured. From each swab, two measurements were taken and means were calculated to determine relative light units (RLU). The RLU was then converted into the amount of ATP by a standard curve constructed with ATP standard solution (LL-100-1, Toyo B-Net Co. Ltd., Tokyo, Japan) in the range of 10^{-16}–10^{-11} mole/100 microliters.

2.6. Statistical analysis

The samples of four pieces of pork meat were selected at random for the storage time period. The regression analysis was carried out to know the relationship between ATP contents and plate count. The raw data had background information; therefore, it was converted by using the 1st and the 2nd derivatives, and the best one was selected.

3. Results

3.1. Plate count

The plate count on the sample meat surface increased with the storage time period. At the outset of the experiment, the initial count was 29 CFU/cm^2 and 84 h after storage it was 3.2×10^7 CFU/cm^2.

3.2. ATP content

The amount of ATP increased with storage time period from the initial amount of 9.2×10^{-5} to 2.8×10^{-10} mol/cm^2 (84 h after storage). A linear relationship was observed between the amount of ATP and the plate count with the determination coefficient (R^2) of 0.95 as shown in Fig. 1.

3.3. Absorption maximum of pure ATP

The transmittance of ATP solutions of different concentration from 1×10^{-4} to 5.85×10^{-6} M are shown in Fig. 2. It shows that the transmittance decreased with an increase in the ATP concentration, and spectra taken for all samples of different ATP concentrations showed that the maximum absorbance related to the decrease in transmittance was at 260 nm (Fig. 2).

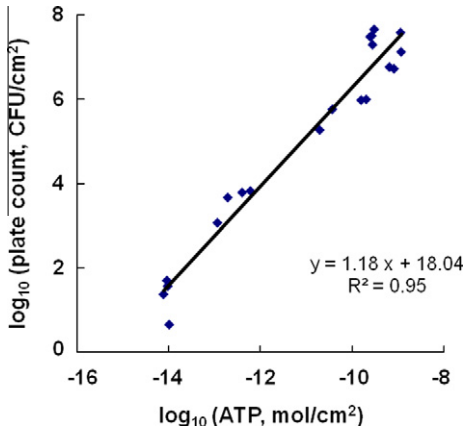

Fig. 1. Relationship between log (ATP) and log (plate count) at different storage time period (from 0 to 84 h).

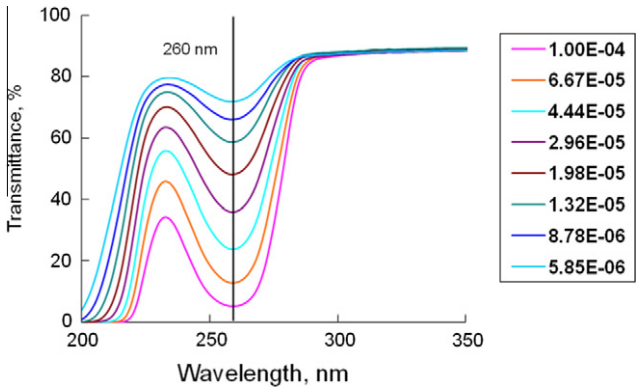

Fig. 2. Transmittance of ATP solutions of different concentrations from 1.00×10^{-4} M to 5.85×10^{-6} M.

3.4. Estimation of ATP and plate count from reflectance

The reflectance spectra obtained at 0–84 h of storage are shown in Fig. 3 in the UV–Vis range (from 240 to 540 nm). There was a very little difference between the reflectance at 0 h and that at 24 h. The reflectance of samples taken at 48, 72 and 84 h, however, showed a decreasing trend with increase in the storage time period.

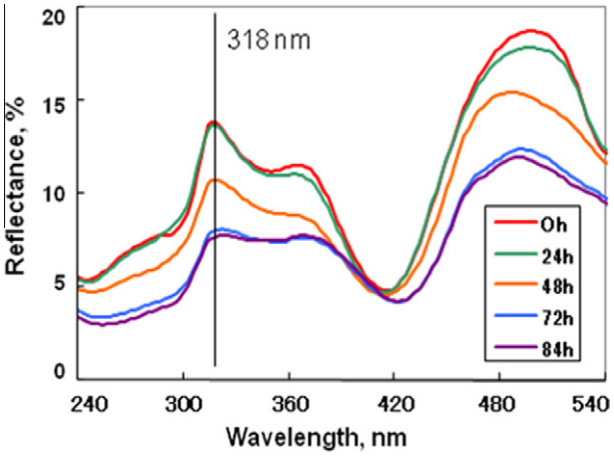

Fig. 3. The reflectance spectra in the UV–Vis range on the pork lion sample surface taken at different time intervals (from 0 to 84 h).

The 2nd derivative of reflectance data selected as the best between 1st and 2nd derivatives of reflectance is shown in Fig. 4. Many upward and downward peaks were observed and the analysis of correlation of peaks at 298, 318, 344 and 374 nm in UV range was conducted. Fig. 5 shows the correlation coefficient between the 2nd derivative of reflectance and log (ATP). This gave a high correlation between the values of the 2nd derivative and log (ATP). Considering bathochromic shift, any of these four wave lengths could be taken as the maximum absorption of ATP.

4. Discussion

Spectroscopic methods have gained importance in the evaluation of food quality attributes during the last decades (Nadai, 1983; Nadai and Mihalyi-Kengyel, 1984). Although NIR spectra reflect several parameters relating to complex quality of food (Williams and Norris, 2001), the information on ATP and/or microorganisms can not be detected in the range of NIR. Therefore, in the present study, UV–Vis was applied ranging from 240 to 540 nm.

4.1. Plate count

In this study, samples were evaluated as fresh until that time when bacterial counts crossed the boundary line of 10^7 CFU/g and no putrid odor could be perceived. After 72 h, the plate count reached the order of 10^7 CFU/g and samples gave off a faint putrid odor. These samples were in the initial stage of spoilage and would be regarded as unacceptable. Plate count is a fundamental index of meat spoilage, and count of 10^7 CFU/g in meat is regarded as unacceptable (Brown, 1982). Detection of the order of 10^6 CFU/g is important as this is achieved just before the meat reaches the unacceptable stage.

Fresh meats generally have a pH range between 5.5 and 5.9 and contain sufficient glucose and other simple carbohydrates to support approximately 10^9 CFU/cm^2. The organisms that grow the fastest and utilize glucose at refrigeration temperatures are the pseudomonas (Gill and Newton, 1977; Jay, 2005; Seymour et al., 1994). At levels of 10^7 CFU/cm^2 off-odors may become evident in the form of a faint 'dairy' type aroma and once the surface population of bacteria has reached 10^8 CFU/cm^2 the supply of simple carbohydrates has been exhausted and recognizable off-odors develop leading to what is known as 'sensory' spoilage (Jackson et al., 1997; Jay, 2005; Stanbridge and Davies, 1998). The development of off-odors is dependent upon the extent to which free amino acid utilization has occurred and these odors have been variously described as dairy/buttery/fatty/cheesy at 10^7 CFU/cm^2 through to a sickly sweet/fruity aroma at 10^8 CFU/cm^2 and finally putrid odor at 10^9 CFU/cm^2 (Adams and Moss, 2007; Dainty et al., 1985).

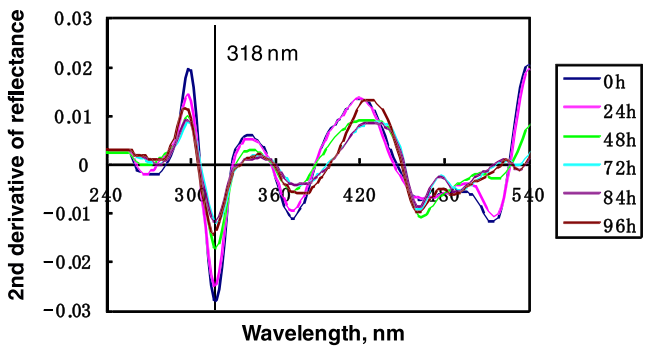

Fig. 4. The 2nd derivatives of raw reflectance spectra from 0 to 96 h.

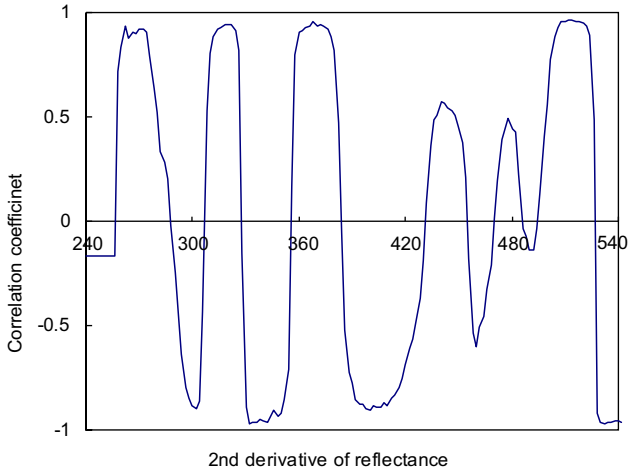

Fig. 5. Correlation coefficient between the 2nd derivative of reflectance and log (ATP).

4.2. ATP content

Fig. 1 shows the linear relationship between $\log_{10}$ ATP and $\log_{10}$ plate count. From this figure both the ATP analysis and the plate count methods were able to assess the hygienence of pork meat samples. The ATP analysis provides only an estimation of the total bacterial count, and cannot differentiate between bacteria (Baumgart, 1993). Theoretically, ATP amounts as low as 100 fg (10^{-13} g) can be measured, corresponding to about 100 bacterial cells. Under practical conditions the sensitivity is about 1000 fg (10^{-12} g), which corresponds to about 1000 bacterial cells or one to two yeast cells (Heeschen et al., 1991). Stressed cells and cells in the stationary growth phase contain less ATP, which also affects the results (Bulte and Reuter, 1985). On the other hand, however, the amount of ATP in a sample provides an estimate of the active microbial population, which is important when considering the shelf life of the product. Stressed cells can also be allowed to resuscitate before the ATP assay (Graumlich, 1985). The enzyme, luciferase, converts the chemical energy provided by ATP into light by a stochiometric reaction. Thus, the amount of light produced is proportional to the concentration of ATP present, which in turn, is directly related to the number of cells in the sample (Bautista et al., 1997). ATP bioluminescence is also useful for monitoring microbial contamination in scalding and chilling tanks within a meat processing operation.

In the ATP bioluminescence assays for carcass contamination and process water quality, microbial cells are removed by filtration before they are lysed to release intracellular ATP. To simplify the method, it would be desirable if the step could be eliminated to allow direct detection of ATP on swabs of the carcass surface, in much the same way as for the ATP bioluminescence hygiene monitoring tests (Griffiths, 1996). However, there would be no way of differentiating ATP from microbial and non-microbial sources using a swab assay, but results would be obtained within 2 min, as opposed to the 10–15 min required when a filtration step is incorporated (Bautista et al., 1997). Siragusa et al. (1996) developed segmented-model statistical approach to determine the lower limits of assay sensitivity and by using this model analyzed in-plant data. According to them, the rapid microbial-ATP test responded in a linear fashion to levels of microbial contamination of >$\log_{10}$ 3.2 aerobic CFU/cm^2 for pork carcasses.

4.3. Absorption maximum of pure ATP

As shown in Fig. 2, the transmittance decreased with an increase in ATP concentration, and different spectra showed the

minimum absorbance at 260 nm. The wave length of 260 nm was in accordance with the maximum absorbance of ATP (259 nm) as previously reported by Bagshaw (2001).

4.4. Estimation of ATP and plate count from reflectance

Reflectance (Fig. 3) showed a decreasing trend with time in the UV–Vis range, although there was a very little difference between the reflectance at 0 h and that at 24 h. To remove the background effect the raw data was transformed by using the 1st and the 2nd derivates. However, the 2nd derivative was chosen because the effect was more clearer in it. The 2nd derivative technique is often used to process NIR data. It helps to separate overlapping absorption bands, remove baseline shifts and increase apparent spectral resolution (Lin et al., 2004), although the derivatives are notoriously sensitive to noise (Tsai and Philpot, 1998). Many upward and downward peaks were observed when the 2nd derivative of raw reflectance spectra for all storage time periods (from 0 to 96 h) was taken (Fig. 4). The analysis of correlation of peaks at 298, 318, 344 and 374 nm was conducted. These selected wavelengths were in the UV range, i.e., less than 400 nm. The greatest differences were obtained for all selected wavelengths, between time 0 and 96 h. The maximum differences between time 0 and 96 h were in the range of 318 nm. This wavelength range could mainly differentiate between samples at 0 and 96 h.

Fig. 5 shows the correlation coefficient between the 2nd derivative of reflectance and log (ATP). This gave a high correlation between the value of the 2nd derivative and log (ATP). Considering bathochromic shift, any of these four wave lengths could be taken as the maximum absorption of ATP.

On the other hand, it is widely known that the spectral absorption by ATP is usually masked by protein absorbance and cannot be exploited in spectroscopic studies (Bagshaw, 2001). However, the graph of correlation coefficient between the 2nd derivative of reflectance and log (plate count) shown in Fig. 6 became very similar in shape to Fig. 5. This indicated that the 2nd derivative of reflectance involved the information of ATP in viable cells. The understanding of this is also supported by the result that the amount of ATP corresponded to the plate count (Fig. 1). From these considerations, the wave length of 318 nm showing the highest correlation coefficient was selected. The linear relationship between the value of the 2nd derivative and log (ATP) for the first 48 h at 318 nm is shown in Fig. 7 with the determination coefficient of 0.89. The similar relationship was also observed between

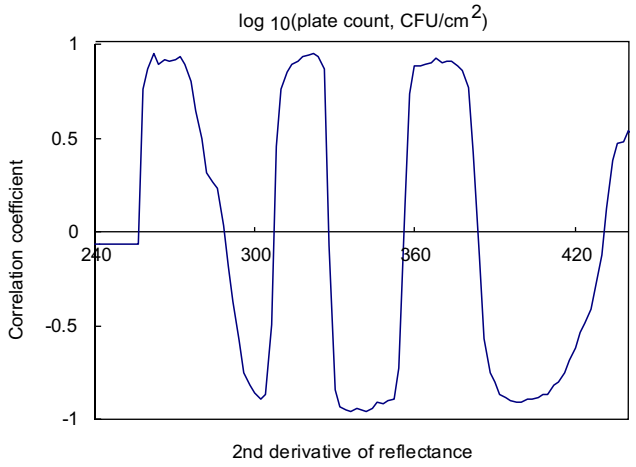

Fig. 6. Correlation coefficient between the 2nd derivative of reflectance and log (plate count).

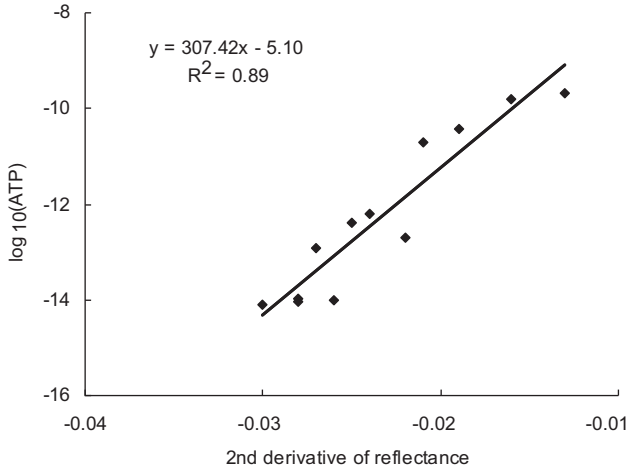

Fig. 7. Linear relationship between 2nd derivative of reflectance and log (ATP) at 318 nm (0–48 h).

the value of the 2nd derivative and log (plate counts) for the first 48 h at 318 nm with the determination coefficient of 0.83. The duration of the first 48 h chosen here means that pork meat samples were fresh. From these results, it is expected that the selection of appropriate wave length could give the real-time monitoring of ATP and/or viable cell count on meat surface by the use of reflectance information.

The plate count gives an estimate of microbial contamination whereas the ATP bioluminescence method used in this study measures total ATP, from both microbial and non-microbial sources, and may be a better measure of the overall cleanliness of the carcass. Therefore, an exact relationship between the two methods should not be expected and results obtained from the two assay systems should be interpreted separately.

Multiple linear regression analysis using more than one reflectance at different wave length is a powerful tool in estimating ATP and/or viable cell count on meat surface, and can lead to higher predictive power. However, such paradigm may lead to overfitting. Accordingly, in this study, only one wave length (i.e., 318 nm) was selected for the prediction of ATP.

5. Conclusions

A real-time detection method for monitoring of ATP and viable cells on meat surface by using reflectance spectra was developed. The data showed that the plate count on the sample meat surface increased and it corresponded exactly to the increase in the amount of ATP during 84 h storage at 15 °C. The linear relationship between the amount of ATP and plate count was supported by its determination coefficient of 0.95. Reflectance showed a decreasing trend with time in UV–Vis range and at the peak of 318 nm, 2nd derivative of reflectance gave a high correlation with log (ATP). As a similar high correlation was also observed between the 2nd derivative of reflectance and log (plate count), it is suggested that the 2nd derivative of reflectance involved the information of ATP in viable cells. From these observations, a linear relationship was given for the estimation of the amount of microbialy-derived ATP on the basis of reflectance analysis of meat surface. Hence, the developed technique can give a powerful way for monitoring of cleanness at a slaughterhouse.

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