Development and Application of μTAS for Detection of Foodstuff Freshness Level

A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Biotechnology
(Doctoral Program in Bioindustrial Sciences)

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ABSTRACT

Foodstuff not only provides energy for human but also has strong associations with human's health. With the improvement of knowledge and development of world trade, people pay more attention to food quality and freshness. Fresh food can provide us essential nutrition; while stale food may have hazardous substances which can cause illness. The conventional methods for food freshness evaluation are either too expensive or need large equipment, μTAS (micro total analysis systems) is a system which can shrink a whole laboratory to a several centimeter chip, and is well known as small sample volume, low consumption of reagents and portability. Therefore, in this study, I developed an acid-base titration μTAS for rice grain freshness evaluation and a sensor-base μTAS for pork freshness evaluation, which are cheap and small-sized.

In the first chapter, the general introduction of the importance, production and consumption of foodstuff were summarized. Moreover, this part also reviewed the former researches in the areas of food freshness evaluation, the development, main technologies and the application of μTAS on food science as well as on other areas, especially the possibility on food quality and safety evaluation.

Rice lipids are easily hydrolyzed to free fatty acids by lipase during storage, in the second chapter, μTAS was developed for detection of fat acidity to evaluate rice freshness. It attained microtitration on a microchip with a main working area of W (11 mm) × L (45 mm). The device was constructed using glass and polydimethylsiloxane plates with flow channels, two injection ports, a volume regulation cell (collection of small plugs), and a φ4.0 mm reaction cell. The small plugs with different volumes are formed by injecting the KOH solution and air to the microchannel simultaneously using microsyringe pumps. As the accumulating of small plugs in the volume regulation cell, large plugs with uniform volume are formed, then, uniform large plug was ejected into

the reaction cell and mixed with the extracted rice sample solution, color change of phenolphthalein, which was added into extracted rice solution as a pH indicator, occurred with the increase in volume of uniform large plugs. Approximately 2 minutes were required for one detection for the fabricated μTAS.

In the third chapter, to verify the performance of device, different varieties of brown and milled rice grains harvested between 2005 and 2010 were used for freshness evaluation. Moreover, to verify fat acidity using μTAS, a conventional neutralization titration method for evaluation freshness was used. According to the results, a good correlation (R^2 = 0.980–0.984) was observed between the fat acidity determined by μTAS and that determined by the conventional method for brown and milled rice grains. Therefore, the developed μTAS can be used for evaluating rice freshness.

Evaluation of nucleotides degradations has been proposed as a rapid and simple method for meat freshness. In the fourth chapter, a W (14 mm) × L (32 mm) μTAS fabricated with four sensors for the determination of pork freshness using freshness indicators, K value (K = {(Ino + Hx)/(ATP + ADP + AMP + IMP + Ino + Hx)} × 100), Ki value (Ki = {(Ino + Hx)/(IMP + Ino + Hx)} × 100) as well as H value (H = {(Hx)/(IMP + Ino + Hx)} × 100), was developed, where ATP, ADP, AMP, IMP, Ino, and Hx are the abbreviations of adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, inosine monophosphate, inosine, and hypoxanthine, respectively. This device was constructed with a sensor layer and a PDMS layer. Four immobilization methods were studied; immobilized and soluble enzyme combination method was the optimum method. The best immobilization condition was investigated, with the best condition, the calibration equations were obtained using standard solutions of ATP-related compounds, and the coefficients of determination for chamber 1 to 4 were 0.887, 0.892, 0.924, and 0.913, respectively.

In the fifth chapter, to verify the performance of device, pork meat stored at

different temperatures as well as from different parts were studied by the developed μTAS. Moreover, to verify the pork freshness by μTAS, UPLC method was used. Investigation of the correlation between K value and Ki value determined by μTAS and by UPLC method showed that, good agreement was observed (R^2 = 0.878-0.924) for K and Ki value respectively, both K value and Ki value could be used for evaluation pork freshness, moreover, the proposed device needed only 2 min for each assay while the UPLC took 10 min. Therefore, the developed μTAS could be used for pork freshness evaluation and was faster and more convenient compared with UPLC equipment. Correlations between K value and Ki value as well as K value and H value were investigated by the developed device, good correlation (R^2=0.992) was observed between K value and Ki value, while for H value, the coefficient of determination was only 0.723. Therefore, the developed μTAS could be used for evaluating pork freshness according to both K value and Ki value.

CHAPTER 1

General Introduction and Literature Review

1.1 General introduction

1.1.1 Importance of food for human beings

Foodstuff plays an important role in people's daily life; they are the source of fats, vitamins, minerals and protein, which are the essential nutrient for human beings. In China, 89 g of protein and 90 g of fat was consumed per person everyday in 2005-2007, in Japan, the consumptions of protein and fat were 92 g and 90 g person^{-1}day^{-1} (FAO Yearbook, 2010). The statistics of per capita consumption of major food commodities in America showed that, 105.7 pounds of red meat, 607.1 pounds of dairy products and 194.5 pounds of flour and cereal products were consumed per capital in 2009 (U.S. Department of Agriculture, Economic Research Service, 2009). Table 1-1 and 1-2 show the consumption of ten major vegetal and animal foods in ten countries and the whole world, rice and wheat consumption takes more than 50% among the ten main vegetal foods, while pork consumption is the largest among the major animal foods in China and Japan in 2005-2007.

Foods not only provide energy for human but also have strong associations with human's health. Fruits and vegetables contain fiber, vitamins and many anti-oxidants like poly-phenolic flavonoids, vitamin-C, anthocyanins, and these compounds can help body to protect from oxidant stress, heart disease, and cancers (Liu et al., 2000; Nomura et al., 2008). Red meat, such as beef, pork, and lamb, contains many essential nutrients necessary for healthy growth and development in children. Some varieties of rice were found to reduce or retard the progression of atherosclerotic plaque development, induced by dietary cholesterol (Ling et al., 2001). Therefore, we need to eat various foods to ensure nutrition intake and avoid illness.

1.1.2 Requirement on food freshness by consumers

The production and consumption of food shows different among countries, to ensure the supply of all varieties of food for consumers, food is now traded and marketed on the global basis. The variety and availability of food is no longer restricted by the diversity of locally grown food or the limitations of the local growing season. Between 1961 and 1999, there was a 400% increase in worldwide food exports. Some countries are now economically dependent on food exports, which in some cases account for over 80% of all exports. On the other hand, the World Bank reported that the European Union was the top food importer in 2005, followed at a distance by USA and Japan. Therefore, products are moving across the countries in today's global supply chain, the degree of freshness of the products was specified by the receiving countries acceptable.

Freshness is a holistic attribute of a food product, with meaning that most often includes how recently produced or harvested a food currently is, to what extent it has been preserved, and it is the opposite of stale or spoiled (Heenan et al., 2009). With the improvement of knowledge, people pay more attention to food quality and freshness, the investigation of food freshness technology company indicates that many consumer have doubts about the reliability and effectiveness of food freshness; 72% of consumers indicate that they discard food at least once a week due to perceived lack of freshness; 67% of consumers estimate that they waste over £10 per month on food thrown away due to perceived lack of freshness; 58% of consumers are willing to pay for a label device that indicates when food is no longer at its freshest. Freshness can not only affect the nutrition value of foodstuff but also induce illness. When foodstuff is not freshness to eat, bacterial may contain in it, which can cause a lot of diseases to human. Therefore, being able to tell when food is fresh is vitally important.

1.1.3 Production and consumption of rice and pork meat

Cereal grains are grown in greater quantities and provide more food energy worldwide than any other type of crop, maize, wheat and rice together accounted for 87% of all grain production worldwide, and 43% of all food calories in 2003, among the three main cereals, rice is the most important staple food for a large part of the world's human population, especially in Asia and the West Indies (Food and agriculture organization of the United Nations, 2006). In Cambodia, for example, 90% of the total agricultural area is used for rice production. In USA, rice consumption has raised sharply over the past 25 years, between 1961 and 2002, per capita consumption of rice increased by 40%.

Meat is one of the oldest foods for human, it has high protein content, can supply the body with the necessary amino acids, and more than 120 kg meat was consumed per capita in USA and Spain in 2003. Moreover, pork is one of the most widely eaten meats in the world, accounting for about 38% of meat production worldwide, although consumption varies widely from place to place (Raloff, 2003); Table 1-3 shows that, nearly 100 million tons of pork were consumed worldwide in 2006. According to the USDA's Foreign Agricultural Service, increasing urbanization and disposable income has led to a rapid rise in pork consumption in China, where 2006 consumption was 20% higher than in 2002, and a further 5% increase projected in 2007.

Considering the importance of rice grain and pork in our daily life, rice and pork was chosen as the subjects for my research.

Table 1-1 Consumption of 10 major vegetal foods (2005-2007)

Dietary energy consumption (kcal person^{-1} d^{-1})

Countries	Rice	wheat	Maize	Pulses	Sugar raw eq.	Potatoes	Cassava	Soybean oil	Palm oil	Rape & mustard oil
Australia	91	541	43	15	402	88	NA	28	137	158
Canada	94	651	117	70	411	121	1	123	2	343
China	802	604	55	12	68	65	4	69	39	42
Japan	606	364	86	16	182	40	NA	114	16	182
Indonesia	1	256	153	189	12	140	8	119	38	8
USA	115	3	129	203	4	197	149	55	109	5
Kazakhstan	134	50	76	42	17	388	19	25	70	1
Ireland	125	31	166	102	23	277	81	27	152	4
France	51	762	88	17	349	115	NA	75	12	76
Italy	132	9	188	52	10	67	179	44	158	3
World	40	11	117	48	7	79	25	38	61	2

Source: FAO year book (2010).

Table 1-2 Consumption of 10 major animal foods (2005-2007)

Dietary energy consumption (kcal person^{-1} d^{-1})

Countries	Bovine meat	Sheep & goat meat	Pigmeat	Poultry meat	Offals edible	Milk(whole)	Cheese	Eggs	Animal fats	Honey
Australia	126	93	109	154	30	186	109	23	153	5
Canada	100	8	115	145	4	62	122	42	236	8
China	25	15	336	53	9	NA	NA	69	40	2
Japan	28	2	90	57	8	84	22	76	37	3
Indonesia	10	3	26	20	5	10	1	18	9	NA
USA	115	3	129	203	4	197	149	55	109	5
Kazakhstan	134	50	76	42	17	388	19	25	70	1
Ireland	125	31	166	102	23	277	81	27	152	4
France	85	21	241	88	23	80	250	55	262	4
Italy	132	9	188	52	10	67	179	44	158	3
World	40	11	117	48	7	79	25	33	61	2

Source: FAO year book (2010).

Table 1-3 Worldwide pork consumption in 2006

Rank	Region	Metric tons (millions)	Per capita (kg)
1	People's Republic of China	52.5	40
2	EU25	20.1	43.9
3	United States	9	29
4	Russian Federation	2.6	18.1
5	Japan	2.5	19.8
6	Others	12.2	N/A
	Total	98.9	N/A

Source: USDA Foreign Agricultural Service, preliminary data for 2006.

1.2 Literature review

1.2.1 Conventional method for foodstuff freshness evaluation

Freshness not only affects food taste but also do harm to human health in some cases. A lot of researches have done to evaluate food freshness. Sensory evaluation is a widely used method for food freshness evaluation, appearance, taste, color and odor were asses by human senses (sight, smell, taste, touch and hearing) to evaluate food quality. It can be applied to evaluate the freshness of meats (Martinsdottir, 1997; Simeonidou et al., 1997; Anastasio et al., 1999); breads (Heenan et al., 2009) as well as fruits and vegetables (Rutkowski et al., 2006; Peneau et al., 2007a). Although, sensory properties of foods contribute very significantly to consumer freshness perceptions, these properties are not easily identified and described as they will vary considerably between different product types (Heenan et al., 2009), on the other hand, there is a high requirement on the assessors. Physical measurements, which include, texture, hardness, microstructure can show food freshness objectively (Heia et al., 1997). Texture properties were shown to be of main importance for apple freshness (Peneau et al., 2007b). Equipments evaluation like HPLC, GC, and spectroscopy are also used in many researches to evaluate food freshness. For example: quantitative determination of amines was carried out by means of HPLC, with spectrophotometric-UV detection, on pre-treated adult bovine and chicken samples to evaluate their freshness, the result showed that, the proposed method is linear in the range of concentrations between 0.01 and 5.0 mug/ml (Vinci and Antonelli, 2002). Furfural is a sensitive indicator of beer staling, researchers have used HPLC to detect furfural, a good inverse correlation between freshness marks and furfural content was observed (Li et al., 2009). A dynamic headspace gas chromatography olfactometry device (DH-GC-MS/8O) was successfully used to reliably identify odorous compounds of European sea bass (Dicentrarchus labrax) after 1, 4 and 15 days of storage in order to find markers of freshness or spoilage

(Leduc et al., 2012). Sinelli et al. (2010) investigated the ability of both NIR (Near Infrared) and MIR (Mid Infrared) spectroscopy to follow minced beef freshness. Freshness of packaged sliced chicken breast was detected using a nondestructive visible and short-wavelength near-infrared (SW-NIR) spectroscopy (Grau et al., 2011). Although these equipments can well show the food freshness, new methods were required because of the high cost of the equipments and the enormous size. Recently, sensors have become a novel and convenient method for food freshness evaluation, such as meats (Lee et al., 2010; Umuhumuza and Sun, 2011), fruits (Shekarriz and Allen, 2008), vegetables (Abdullah et al., 2008) and cereal products (Botre and Gharpure, 2006). These sensors are always easy to make, and with low cost, therefore, they are expected to be a promising method in food freshness evaluation. Some other technique have also been used to assess food freshness, such as pH value, microorganism numbers for dairy, colorimetric method for meat and rice (Matsukura et al., 2000; Srirangsan et al., 2010); fluorescence imaging for rice grains (Hachiya et al., 2009).

1.2.2 Introductions of μTAS

Micro total analysis systems (μTAS), also called "lab on a chip", or miniaturized analysis systems, was originally proposed by Manz et al. (1990). It is a system which can shrink a whole laboratory to a several centimeter chip; therefore, sample preparation, separation, reaction and detection can be realized on one chip. The advantages of μTAS are well known: small sample volumes, low consumption of reagents and portability.

The main technologies for fabrication a μTAS include: structure design, bonding techniques, surface modification and so on. Structure design should consider the viscosity of the reagents, the principle of the detection, the dimensions (including the depth, width and length) of flow channels and reaction chambers of flow channel. Van der Graaf et al. (2005) studied the formation of plugs on chip using a T-shaped microchannel junction. In order to minimize band broadening and stretching, Fiechtner

and cummings (2003) proposed a new methodology to design flow channels for ideal electrokinetic flow. Bonding is also an important technology in fabrication of micro total analysis systems. Ito et al. (2002) developed a simplified glass bonding technique utilizing a water glass solution. A novel heating technique for silicon-silicon bonding using electromagnetic radiation was studied by Thompson et al. (2002). Kim et al. (2010) stated an optimized bonding method to bond poly (methyl methacrylate) (PMMA) and polydimethylsiloxane (PDMS) substrates using silane primer. PDMS has been widely used in μTAS, in order to control the hydrophilicity and nonspecific adsorption of biomolecules on their surfaces generally, surface modification was needed when the devices used in biological applications. Hamda et al. (2007) reported a simple but reliable method of applying a surface coating of biomimetic polymer to PDMS surfaces by the combinative use of photochemical surface oxidation by VUV irradiation and subsequent coating of phospholipid copolymer containing 2-methacryloyloxyethyl phosphorylcholine (MPC) and 3-methacryloxytriethoxysilane (METESi). Hydrophobic and other interactions can cause undesired adsorption of analyte onto the microchannel walls leading to the loss of reagent and the nonuniform distribution of analyte, therefore, surface modification was studied to optimize the surface of microchannel to solve these problems. Hu et al. (2002) demonstrated a one-step procedure to covalently link polymers to the surface of PDMS microchannels by ultraviolet graft polymerization. Micro PCR chips were coated with Parylene film, which has low permeability to moisture and long-term stability, to solve the problem caused by the porosity of PDMS (Shin et al., 2003). Surface modification can help to improve the reliable and repeatable of the device.

The analytical procedures for a μTAS always include: sample preparation, injection, fluid and particle handling, mixing, separation and detection. The mainly used detection methods are shown in Table 1-4. A micro optical system integrated with a micro fluid device is a convenient way to realize biochemical reaction and detection on

one chip. A miniaturized and sensitive optical system has been developed for micro total analysis systems based on laser induced fluorescence detection (Yang et al., 2000). In recent years, chemiluminescence (CL)-based detection coupled to capillary electrophoresis as separation technique has attracted much interest (Garcia-Campana et al., 2009), a micro total analysis systems with chemiluminescence detection and its application to detection of cancer marker was stated by Tsukagoshi et al.(2005), a chemiluminescence (CL) micro-total analytical system is also described for the analysis of codeine (Greenway et al., 2000). Sensors were also used for detection in micro analysis systems because of its fast and easy for crominiaturization. A capillary electrophoresis (CE) micro-analytical chip has been designed and fabricated with integrated electrodes for sample injection, separation and electrochemical detection using standard fabrication technology (Virdi et al., 2008). An electrochemical immunosensor incorporated in a micro fluidic cell for quantification of citrine (CIT) mycotoxin in rice samples was developed, the result showed that, electrochemical immunosensor showed a higher sensitivity and reduced analysis time compared to other analytical methods such as chromatographic methods (Arevalo et al., 2011).

The application of μTAS has grown exponentially in the past decade, clinical diagnostics, environmental concerns, immunoassays, proteins as well as DNA separation and analysis have been studied and realized using μTAS. Cakal et al. (2010) developed a μTAS for catecholamines analysis in which preconcentration, separation, and determination steps were integrated on a microchip, the microchip could be used more than 50 times if mechanical problems such as plugging or fracturing did not occur. An electronic blood collection system employing a painless needle was developed for home medical diagnosis, both the healthcare chip and hepatic function examining chip were studied based on the introduction of plasmas separated from a trace amount of blood (Oki et al., 2004). For the past two decades intensive research has been carried out on environmental sensors. Nevertheless, the applicability of such devices has been

obstructed by harsh work conditions and the complexity of the sample matrices, μTAS focused on the integration of sample pretreatment steps was studied (Saint-Pierre et al., 2005). DNA analysis is also one of the primary applications of μTAS technology (Sun and Kwok, 2006). An SPE microfluidic chip for DNA extraction by using porous silicon dioxide as the solid phase matrix has been developed (Chen et al., 2006). Moreover, as Table 1-5 shows, μTAS has also been used by companies in USA, Japan, Germany and so on.

1.2.3 Applications of μTAS on foodstuff evaluation

As I stated in 1.2.2, μTAS has been applied on many areas, Table1-5 shows that, μTAS has also been widely studied on evaluation of foodstuffs in USA, Japan and Germany. Foodstuff evaluations include food safety, nutrition and freshness. Quantification of citrine (CIT) mycotoxin in rice samples was established by an electrochemical immunosensor incorporated in a micro fluidic cell, the results showed that, electrochemical immunosensor showed to be a very useful tool to determine CIT in samples of cereals, mainly rice samples (Arevalo et al., 2011). L-glutamate is one of the most important amino acids to analyze in food safety, two microfluidic systems have been developed for specific analysis of l-glutamate in food based on substrate recycling fluorescence detection (Laiwattanapaisal et al., 2009).

1.3 Objectives of this study

Freshness of foodstuff is an important index to food quality, it can not only affect the taste of food, but also have close connection with our health in some cases. Rice and pork are two essential foods in many countries, like China, Japan and USA. It is necessary to evaluate their freshness to provide tasty and safety products to consumers. The traditional methods are either expensive or need large amount of reagents. On the contrary, μTAS has been applied to realize conventional detection in many areas,

moreover, application of μTAS on food freshness is rare, therefore, in this study μTAS was used for food freshness especially rice and pork meat, which are two main food in daily life.

This study was under taken with the following objectives:

1) To design and fabricate a titration-based μTAS.
2) To apply the titration-based μTAS on rice freshness evaluation and compared with conventional fat acidity method.
3) To design and fabricate a μTAS for pork freshness evaluation using enzyme sensors for detection.
4) To study the best immobilization method and conditions for the enzymes.
5) To apply the developed enzyme based μTAS on pork freshness evaluation and compared with conventional UPLC method.

Table 1-4 Detection methods used in micro total analysis systems

Detection method	Merit	Drawback
Laser induced fluorescence	High detection sensitivity	Fluorescence labeling may change the biochemical activity of analyte, therefore, effect the reliability of the results
UV absorbance	Universal optical detector	Low sensitivity; high requirement on chip materials
Chemiluminescence	High detection sensitivity; no requirement on light source, easy for crominiaturization	Has special requirement on detection reservoir and efficient mixture of chemiluminescence reagent and analyte
Electrochemical	High detection sensitivity	The analyte should have electrochemical activity; bad reproducibility
Sensor	Fast and easy for crominiaturization	Biosensors easy to lose stability during storage.
Mass spectrum	Can provide basic structure and quantitative information for bimolecular	The equipments are large and Expensive

Lin, B. C. and Qin, J. H. (2008). Illustrate microfludic chip laboratory. Beijing, Science Press.

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Table 1-5 Companies producing and commercializing microfluidic devices and systems for applications in agri-food industries

NO.	Company Name and Location	Technology/Application
1	Affymetrix Inc., Santa Clara, CA, USA	Biochips for sequencing the genomes of cattle that relate to commercially valuable traits such as disease resistance and leanness of meat.
2	Qiagen, Hildon, Germany	Sample and assay technologies for food, animal pathogen testing
3	Caliper Life Sciences Inc., Hopkinton, MA, USA	Labchip GX platform for high throughput screening and predictive assessments of biological and food product quality
4	Dupont, Wilmington, DE, USA	Qualicon food safety sensor for testing food-borne bacteria using capillary electrophoresis
5	Epigem Ltd, Redcar, UK	Fluency microfluidic chips for biochemical monitoring of food, soil and water
6	Nanoterra, Inc., Cambridge, MA, USA	Portable analytical systems for food safety monitoring, pathogen detection in water, and for creating monodisperse droplets, foams, and colloids in food industries

Neethirajan, S., et al. (2011). Microfluidics for food, agriculture and biosystems industries. Lab on Chip. 11(9). 1574-1586.

CHAPTER 2

Development of μTAS for Rice Freshness Evaluation

2.1 Introduction

As stated in chapter 1, rice is the most important staple food for a large part of the world's human population, it sustains two-thirds of the world's population. In the distribution process, only a small proportion is eaten immediately, while a large amount is stored to guarantee a constant supply to the market as well as to avoid rice shortage in case of emergency. However, many physical, chemical, and biological changes occur during storage, including decomposition of lipids, starch, and protein, which directly results in the decrease in freshness and taste (Juliano et al., 1964; Zhu, 1999; Zhou et al., 2001; Ohno and Ohisa, 2005; Tananuwong and Malila, 2011). Therefore, it is necessary to evaluate rice freshness to provide excellent quality rice to consumers.

Many methods have been developed for evaluating rice freshness. These include the fat acidity method, the TTC (2,3,5-Triphenyl-2H-tetrazolium chloride) method, the KI (potassium iodide) coloration method, Thin-Layer Chromatography and Flame-Ionization Detection System and the near-infrared spectroscopic method (Zhu, 1999; Nishiba et al., 2000; Fan et al., 2009).

Of the possible indicators, lipids are closely related with the aging process. Lipids are easily hydrolyzed to free fatty acids by their specific enzymes during storage (Zhou et al., 2001; Tran et al., 2005). Therefore, the level of fat acidity can be used as an indicator of rice freshness. To detect fat acidity, indicator methods, pH meters, and colorimetric methods have been used (Shimizu et al., 1998; Xu et al., 2007; Yan, 2007). In particular, the conventional method for determining the fat acidity of cereal products based on titration has widely been used. However, this

method requires a handful of rice grains for one measurement. Furthermore, the determination of the end point by a color change tends to be inaccurate because of the large volume of the solution to be titrated. On the contrary, a micro total analysis systems (μTAS, i.e., microfluidic system) (Neethirajan et al., 2011) can process minute volumes of fluids in flow channels with dimensions of tens to hundreds of micrometers (Whitesides, 2006). They can perform the same operations conducted for conventional analyses, but with very low consumption of reagents and an extremely short reaction time (Yang et al., 2010). Of these μTAS, plug-based devices are a promising platform (Tice et al., 2003; Sassa et al., 2008; Shimizu et al., 2009). Therefore, in this study, a plug-based μTAS was developed on a microchip and was used for neutralization titration as well as for detecting the level of fat acidity in rice grains. The performance of μTAS developed was evaluated by comparing the fat acidity value detected by this device with that detected by the conventional method.

2.2 Materials and methods

2.2.1 Materials and equipment

Reagents and equipment used for fabrication of the device and for titration were obtained from the following commercial sources: glass wafers (No. 7740, 3 in., 500-μm thick) from Corning Japan (Tokyo, Japan); a thick film photoresist, SU-8 from Microchem (Newton, MA, USA); precursor solution of polydimethylsiloxane (PDMS), KE-1300T from Shin-Etsu Chemical (Tokyo, Japan); and the water repellant Fluorosurf (FS-1060TH-2.0) from Fluorotechnology (Aichi, Japan). All solutions were prepared with distilled deionized water.

2.2.2 Fabrication of the device

The device was constructed with a glass substrate that was bonded to a PDMS substrate after the formation of internal structures (Fig. 2.1). These structures were formed in the PDMS substrate by replica molding using a template formed with a thick film photoresist (SU-8). Take the optimum device for example. First, the photoresist was spin coated on a clean glass wafer using a spin coater (1H-D7; Mikasa Corporation, Japan). Next, another 1.5 g of SU-8 was weighed and uniformly coated on to the wafer to thicken the layer. The wafer was then baked on a hot plate for 5 min at 65 ℃ and for 2 h at 95 ℃. After cooling down, the photoresist layer was exposed to UV light on a mask aligner (MA-10; Mikasa, Japan) and was developed in an SU-8 developer. As a result, patterns for the flow channels were formed. A precursor solution of PDMS was dropped onto the template after bubbles were removed, and PDMS was cured by baking for 30 min at 80 ℃. Through-holes (diameter, 4.0 mm) were punched into the PDMS substrate to form inlets and a titration cell. Finally, PDMS and glass substrates were bonded together after the PDMS surface was treated with air plasma (Model BP-1; Samco Company, Japan). As a result, the reaction cell, volume regulation cell, flow channels, and two ports for a titrant solution and for air were formed on the device (Fig. 2.1). The height of the internal structures was 150 μm. The width of the branched flow channels for the titrant solution and air was 300 μm and 600 μm, respectively. The flow channels were connected to a 200-μm-wide flow channel, which was also connected to the volume regulation cell. A 500-μm-wide flow channel extended from the volume regulation cell to the reaction cell. Because the glass plate was hydrophilic, the walls of the flow channels were coated with a hydrophobic layer (Fluorosurf) to ensure stable movement of plugs after bonding of PDMS and glass plates. Two microsyringe pumps (BS-MD1001; Bioanalytical Systems, Inc., USA. and MSPE-3; As ONE Corporation, Japan) were connected to the device by silicon tubes to control the injection of air

and the titrant solution (Fig. 2.2).

2.2.3 Optimization of the structures and dimensions of the device

The dimensions of the channels, including depth, width and length can affect the flow of the reagent in the flow channels, as a result, affect the forming of plugs. in this study, three different shapes and sizes of devices were studied (Fig. 2.4).

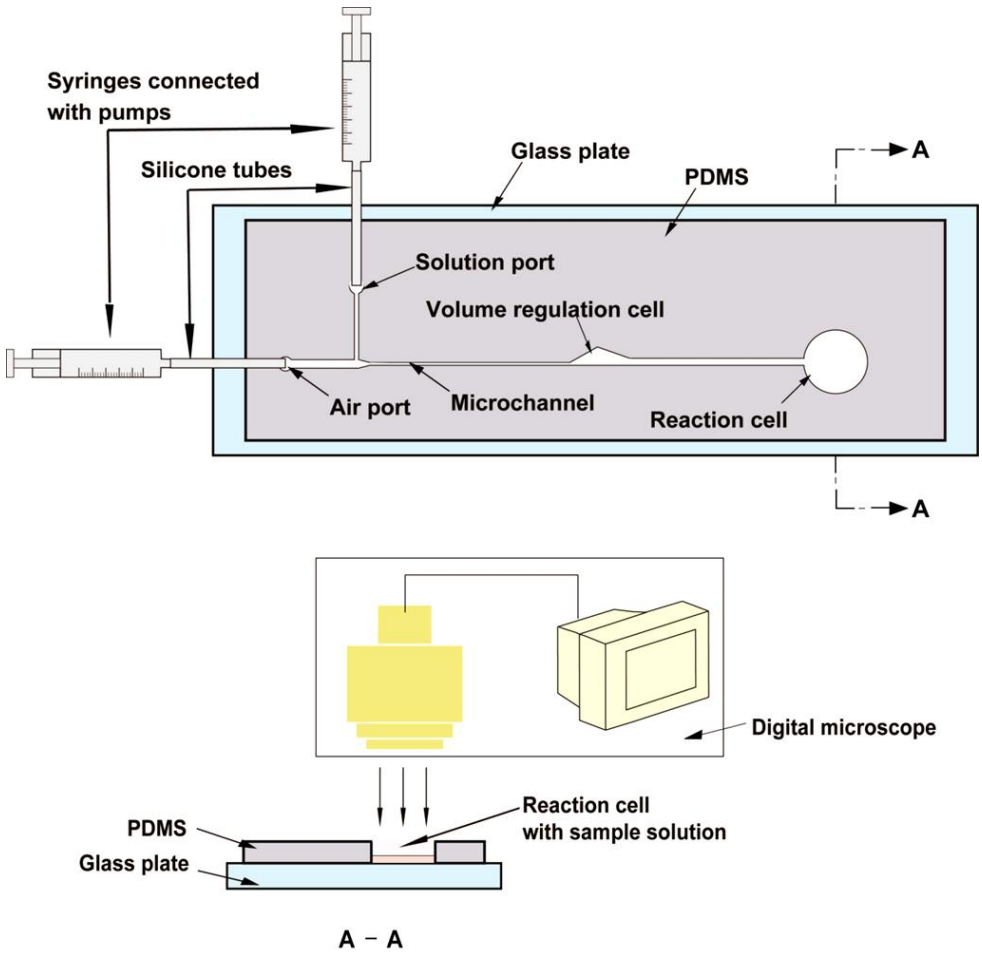

Fig. 2.1 Structure of μTAS for rice freshness evaluation.

Small plugs were formed at the T-junction. Uniform plugs formed in the volume regulation unit were injected into the titration chamber. A-A shows the profile of the titration chamber

2.2.4 Formation of plugs and on-chip titration

A unique feature of our device is the volume regulation cell that forms a row of plugs of uniform volume from small fragments of a solution of uncontrolled volumes (Fig. 2.2). The small fragments are formed by simultaneously injecting a titrant solution and air into a T-junction using two microsyringe pumps, with a flow rate of 100 $\mu L\ min^{-1}$ for air and 0.5 $\mu L\ min^{-1}$ for the solution. However, the volumes at this point are far from uniform. The volume of the volume regulation cell is much larger than that of the small fragments. Therefore, until a certain number of fragments have accumulated and the plug thus formed has closed the volume regulation cell, pressure is released through a space made between the growing plug and the walls. The plug remains in position until the total volume exceeds a threshold determined by the size and shape of the volume regulation cell and the flow channel is closed, at which point pressure is again applied to the plug. The plug is then ejected from the volume regulation cell and is transported to the reaction cell located in the lower stream (Fig. 2.2).

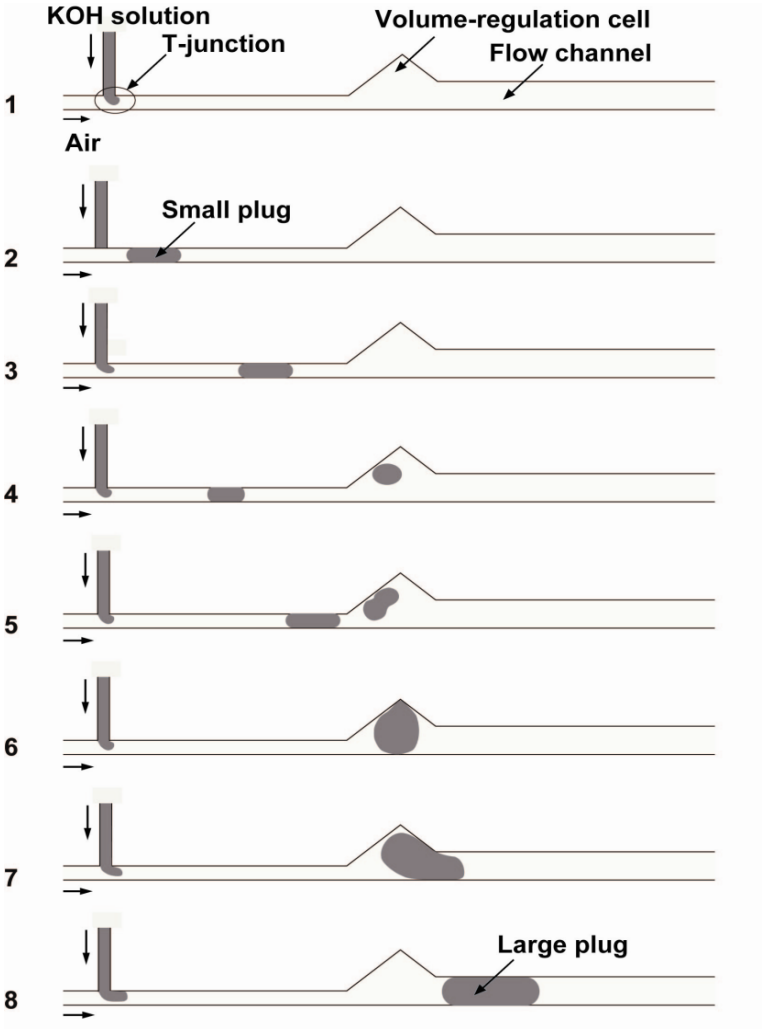

Fig. 2.2 Formation of uniform plugs.

(1,2) Small plugs are formed at the T-junction by the simultaneous injection of air and KOH solution. (3–5) New small plugs are formed continuously and are injected into the volume regulation cell. (6) Small plugs merge in the volume-regulation cell. (7,8) A large plug formed in the volume regulation cell is ejected, and new small plugs will be formed at the T-junction repeatedly.

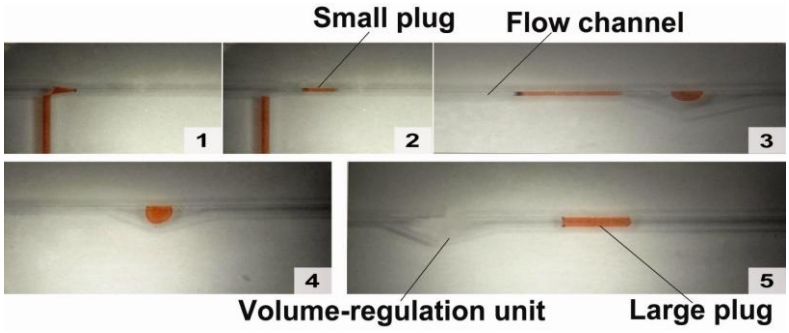

Fig.2.3. Formation of plugs in μTAS using food dye.

(1-2) Formation of small plugs at the T-junction. (3-4) Accumulation of the plugs in the volume-regulation unit. (5) Ejection of a large plug from the volume-regulation unit. A food dye (Red No.102) was used to visualize the formation of the plug.

2.3 Results and discussion

2.3.1 The size and shape of the flow channels

A critical part of the device is the volume-regulation unit. The dimensions and shape of the unit, as well as the length and width of the flow channels, were optimized. Figure 2.4 shows three devices with different structures of inlets, flow channels, and volume-regulation unit. With A and B, fragments were formed smoothly. However, small fragments tended to be left on the wall of the volume regulation unit. With C, although having a similar structure with the optimum structure except for the difference in inlets, fragments of solution were not steady enough, and could not be ejected completely in some case. The optimized device (Fig.2.1) could avoid such problems, which was used as the final device in later experiments.

2.3.2 Influence of the flow rates of air and KOH ethanol solution

The ratio of the flow rates of air and KOH ethanol solution influences the length and velocity of the plugs formed in the volume regulation cell. Therefore, for the best flow channel structure, the KOH ethanol solution and air are injected into the branched flow channels at different flow rates to optimize the formation of the plugs.

Figure 2.5 compares the dependence of the relative standard deviation of the volume of the plug formed in the volume regulation cell and the fragments formed at the T-junction on the ratio of the flow rates of air and KOH ethanol solution. The relative standard deviation of the processed plugs formed in the volume regulation cell was approximately half of that of the unprocessed small fragments formed at the T-junction, which indicates that the volume regulation cell worked as expected. The relative standard deviation was smallest at a ratio of 63 when the flow rate was 50 $\mu L\ min^{-1}$ for air and 0.8 $\mu L\ min^{-1}$ for the solution, and it tended to saturate at higher ratios. However, the average volume of the plug was 196 nl when the ratio was 63 and

154 nl when the ratio was 200. Because a smaller plug volume is preferable to improve the resolution of the titration, the ratio was set at 200 in subsequent experiments. Furthermore, 70 fragments of solution were formed under a ratio of 200 to investigate the reproducibility of the device; 89% of the plugs had volumes between 0.15 and 0.20 μL (Fig. 2.6).

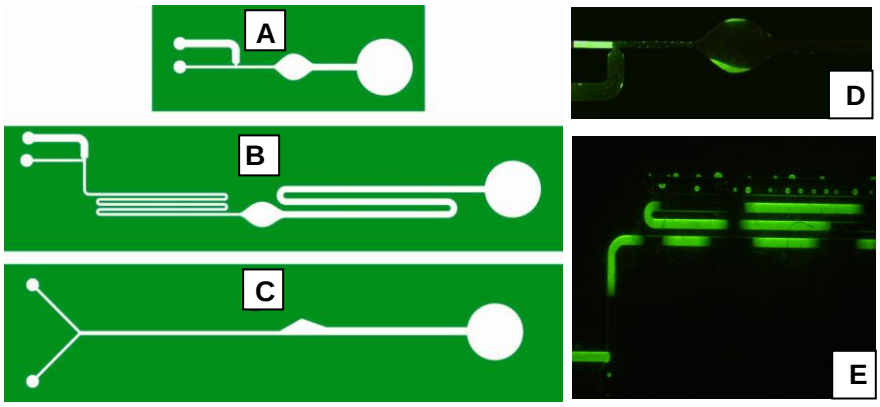

Fig.2.4 Structure of inlets, microchannel and volume-regulation unit.

A, B, C show devices with different length, inlets, channel structure and shapes of volume-regulation unit. D, E show the fluorescence imagine of solutions in structure A and B.

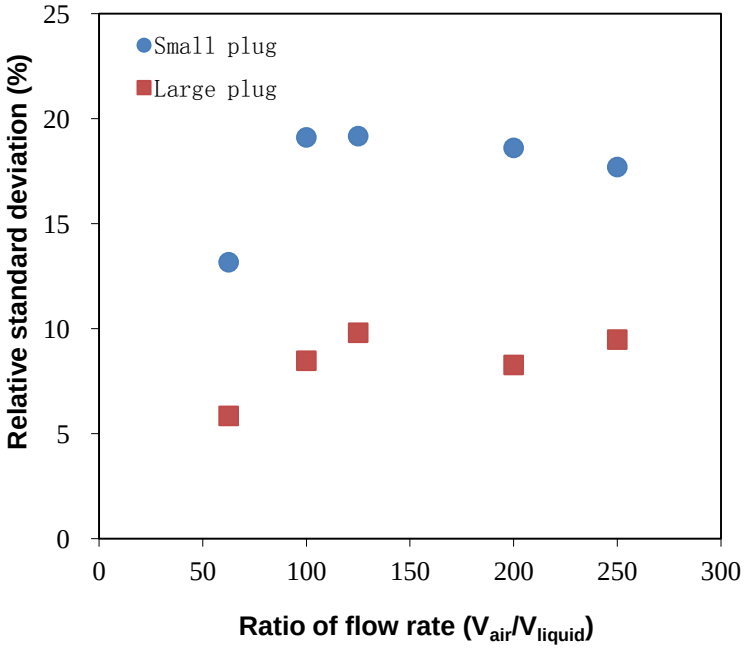

Fig. 2.5 Relationship between the ratio of the flow rate and the relative standard deviation of plug volume.

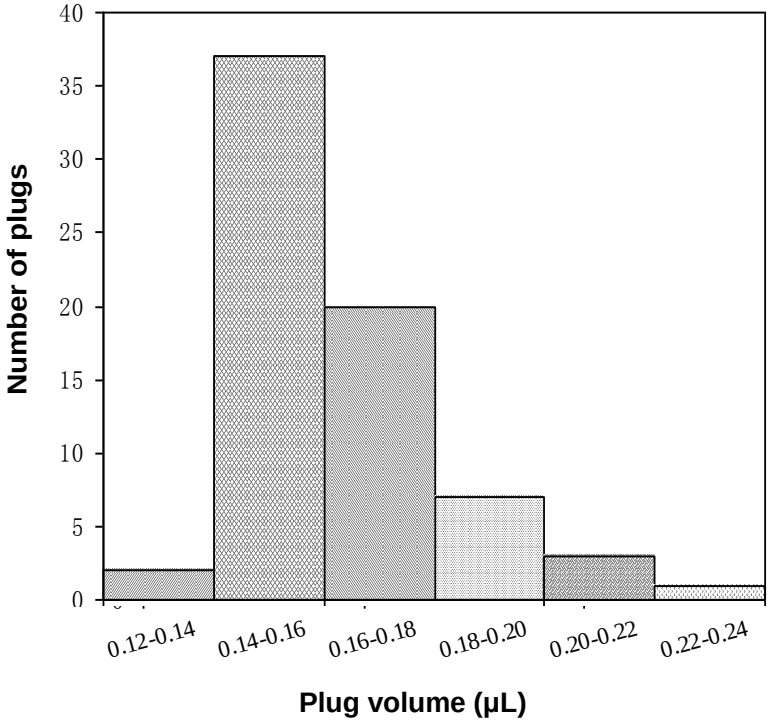

Fig. 2.6 Distribution of the volume of plugs formed in the volume regulation cell

2.4 Conclusions

A μTAS that can conduct on-chip acid–base titration was developed. The dimensions and shape of the unit, as well as the length and width of the flow channels, can affect the formation of plugs and the volume of the large plugs. The volume regulation cell in the device can form plugs of uniform volumes. The relative standard deviation of the volume of the plugs formed in the volume regulation cell is much smaller than that of the fragments formed at the T-junction. The volume of the plug can be adjusted by changing the ratio of the flow rates of the titrant solution and air. The coefficient of variation of the formed large plugs was less than 10 % when investigation of 10 large plugs, which showed that the accuracy was good. These characteristics of μTAS facilitated titration of free fatty acid to evaluate rice freshness.

CHAPTER 3

Comparison of Fat Acidity Assessed by μTAS and Conventional Titration Equipment for Rice Freshness Evaluation

3.1 Introduction

In chapter 2, a μTAS was developed to evaluate rice freshness, in this chapter, I will use the developed μTAS and the conventional fat acidity method for rice freshness eavaluation. Rice grains harvested in different years, with different species were studied using both fat acidity method and μTAS. The correlation between these two method was also investigated using rice harvseted in different years.

3.2 Research methods

3.2.1 Experimental materials and equipment

Rice samples

Both brown and milled rice grains were used in our research. Rice grains harvested between 2005 and 2010 stored at 5 ^{o}C were used to determine changes in fat acidity based on the harvest year (Table 3-1). The fat acidity of four different varieties of rice grains was tested: *Oryza sativa* L. subsp. *Japonica* cv. Koshihikari, Hitomebore, Yumehitachi, and Kinuhikari. The rice was harvested in 2010 in Ibaraki, Japan. Milled rice was stored in polyethylene bags at 5 ℃ in a refrigerated showcase (MPR-311DR; Sanyo, Japan) for approximately one week after milling. According to the equation explained later (Park et al., 2001), degree of milling were calculated to be approximately 10%:

Degree of milling (%) = (1 − (weight (g) of milled rice/weight (g) of brown rice)) × 100. Eq. (3-1)

Reagents and equipment

Potassium hydroxide, phenolphthalein and ethanol, bought from Wako, Japan. Ministirrer TR-100 (Pasolina company, Japan), rice polisher VP-30 (Yamamoto company, Japan), burret meter, 10 mL (Sibata Co. ltd, Japan).

Table 3-1 Rice grains used in this study

No.	Producing prefecture in Japan	Varieties	Harvested year
1	Unknown	Unknown	2005
2	Niigata	Koshihikari	2006
3	Niigata	Koshihikari	2009
4	Ibaraki	Koshihikari	2010
5	Ibaraki	Hitomebore	2010
6	Ibaraki	Yumehitachi	2010
7	Ibaraki	Kinuhikari	2010

3.2.2 On-chip acid–base titration

During testing of the device, the reaction cell was filled with 1.5 μL of 1 mM, 2 mM, 4 mM, 8 mM, and 10 mM HCl solution (1% phenolphthalein solution was added as the pH indicator). Two microsyringe pumps filled with air and 10 mM KOH solution were switched on at different flow rates. Fragments of the KOH solution were formed and transported downstream. Uniform plugs formed in the volume regulation cell were eventually injected into the reaction cell to titrate HCl. The color change that occurred in the reaction cell was observed under a digital microscope (VHX-1000; Keyence, Japan). The volume of the KOH solution used for titration was measured by counting the number of the plugs injected into the reaction cell.

3.2.3 Procedures for evaluating rice freshness

Different varieties of brown and milled rice grains harvested in different years were evaluated. The fat acidity was expressed as mg KOH consumed to neutralize the free fatty acid in 100 g of dry matter (Mestres et al., 2003). The value was calculated using the following equation:

$$\text{Fat acidity (mg KOH/100 g dry matter)} = 100\,(V_1 - V_0)\,CD\,(B/A)/(mE\,(100 - W)) \quad \text{Eq. (3-2)}$$

Here, V_1 is the volume of the KOH ethanol solution consumed in the neutralization titration (mL), V_0 is the volume of the KOH ethanol solution consumed to titrate the blank solution (mL) (i.e., the absolute ethanol solution used to replace the rice extract solution), C is the concentration of the KOH ethanol solution (mol L^{-1}), D is the molar mass of KOH (g mol^{-1}), m is the weight of rice grains used for extraction (g), B is the volume of absolute ethanol used for extracting free fatty acid from the rice flours (mL),

A is the volume of rice extract used for titration (mL), *E* is the conversion to a 100 g sample (g), *W* is the moisture content of the rice flour (i.e., the ratio of the weight of the dried rice flour to the original rice flour). In our experiments, the values of *A*, *B*, *D*, and *E* were 25, 50, 65.1, and 100, respectively.

1) Preparation of extracts of rice samples

Before using μTAS, rice extraction solution should be prepared first. When preparing the samples, approximately 15 g of the rice samples listed in Table 3-1 was weighed and pulverized to avoid any loss of weight during pulverization. A total of 10 g rice flour was then weighed and transferred to a flask to which 50 mL of ethanol was added, and the mixture stirred for 10 min. Next, the extract was filtered, and 25 mL of the solution was collected; 50 mL of distilled water was then added to the 25 mL of solution and mixed well. For the blank test, 25 mL of absolute ethanol was used instead of the rice solution.

2) Preparation of reagent for μTAS

The size of the whole device was smaller than the conventional burret and the conical beaker used for reaction. Therefore, the amount of rice extract and the best concentration of KOH-ethanol solution was investigated. Considering the different fatty acid values of the rice grains, the optimum amount of rice extract for the brown and milled rice was decided separately. For brown rice, the reaction cell was filled with 0.4 μL of the rice extract containing 1% phenolphthalein solution as the indicator. Free fatty acid was then titrated with 9.6 mM KOH ethanol solution. For milled rice, 1.2 μL of the rice extract was used, and the concentration of the KOH ethanol solution was 4.8 mM. This is because the color change for the milled rice harvested in 2010 was extremely fast when 9.6 mM KOH ethanol was used. An enormous increase in the volume of the rice extract solution decreased the clarity of the results because of the influence of prolamine, which made the solution a little turbid.

3) Connection of the μTAS and detection

As stated in chapter 2, the microsyringe pumps used to inject the KOH ethanol solution and air into the device were turned on after the rice extract solution was injected into the reaction cell. Large plugs were formed and injected into the reaction cell where they mixed with the rice extract. A color change was observed when all of the free fatty acid in the rice extract solution was neutralized by KOH ethanol. The two microsyringe pumps were turned off at this point.

3.3 Results and discussion

3.3.1 Acid–base titration

The titration of the different concentrations of HCl was performed using KOH as the titrant. For 10 mM HCl, the color change was not obvious with the first eleven plugs, indicating that the reaction had not reached the end point. The color change was observed when the twelfth plug was injected; this indicates that the reaction had reached the end point. As the number plugs increased, the color change continued and was eventually saturated. In the investigation to determine the volume of KOH that corresponded to the different concentrations of HCl (Fig. 3.1), the volume of the KOH solution increased as the HCl concentration increased, and a good correlation was observed (R^2 = 0.971). These results demonstrated that the device can be used for acid–base titration.

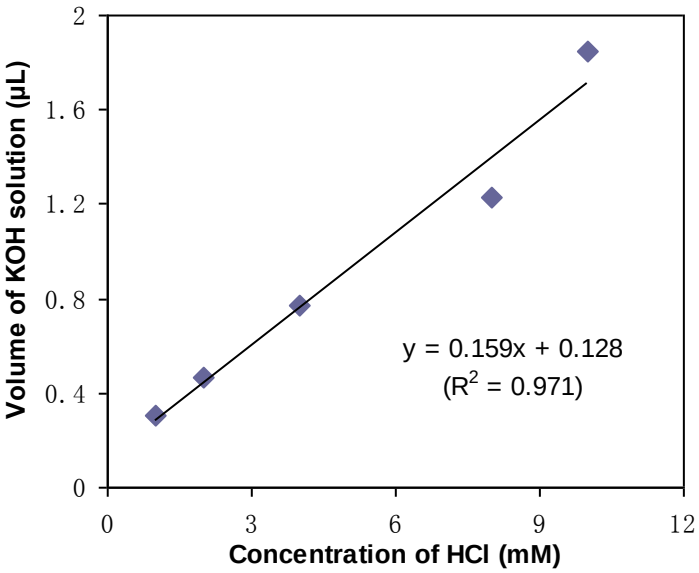

Fig. 3.1 Calibration curve of acid-base titration assessed by μTAS.

3.3.2 Rice freshness evaluation by μTAS

Figure 3.2 shows the fat acidity of brown and milled rice harvested in different years as assessed by μTAS. With brown rice, a sudden color change was observed at an early stage with rice harvested in 2009 and 2010, indicating that the amount of fatty acid was small. The lipid was stable during storage at 5 ℃ for less than 14 months. In addition, Zhou et al. (2001) concluded that the lipid content of brown rice was stable during storage for 12 months at 5 ℃. On the other hand, the initiation of the change was delayed with older rice samples, indicating the accumulation of fatty acid within the extract. For milled rice, the volume of KOH ethanol solution consumed for rice harvested in 2005 was larger than that for other years, which indicated that the accumulation of free fatty acid in milled rice flour pulverized from brown rice harvested in 2005 was greater.

Furthermore, fat acidity was investigated with samples of different rice varieties harvested in 2010 in Ibaraki Prefecture. There was no significant difference in fat acidity among the brown rice varieties harvested in the same area, except for the Koshihikari cultivar (Fig. 3.3). Moreover, when the different varieties of milled rice grains harvested in 2010 in Ibaraki Prefecture were assessed, only an extremely slight difference was observed compared with brown rice.

When different varieties of brown and milled rice harvested in different years were compared (Figs. 3.2, 3.3), levels of fat acidity were greater for brown rice than for the corresponding milled rice. This phenomenon was also observed by Tran et al. (2005). This may because brown rice contains pericarp and has more lipid than milled rice, which lacks pericarp.

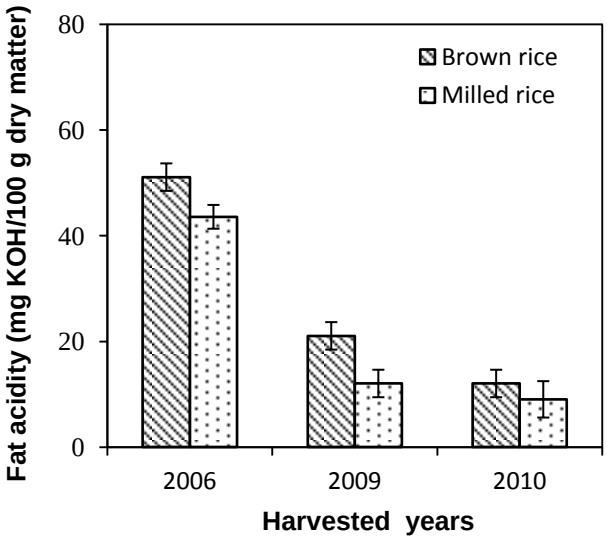

Fig. 3.2 Fat acidity of brown rice (rice samples numbered 1 to 4 in Table 1) and milled rice produced in different years.

Labeled error bars indicate the standard deviations for each measurement (n = 3).

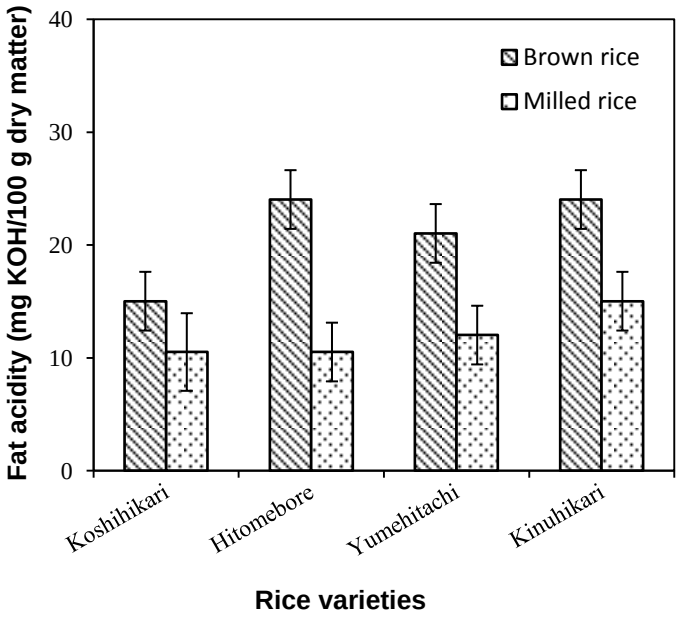

Fig. 3.3 Comparison of the fat acidity of different varieties of brown and milled rice produced in Ibaraki Prefecture (2010).

Labeled error bars indicate the standard deviations for each measurement (n = 3).

3.3.3 Comparison of μTAS and conventional titration equipment

Brown rice harvested in different years (2005, 2006, 2009, and 2010) as well as their corresponding milled rice grains (stored at 5℃ for one week) was assessed (in triplicate) to compare values obtained using μTAS based on titration and those obtained by the conventional titration method (Fig. 3.4). Regression equations and correlation coefficients of the two methods with brown and milled rice grains were as follows: $y = 0.955x + 2.390$ ($R^2 = 0.980$), $y = 1.212x - 4.312$ ($R^2 = 0.984$). Therefore, it is possible that rice grain freshness can be evaluated using μTAS, but the device has to be optimized in future.

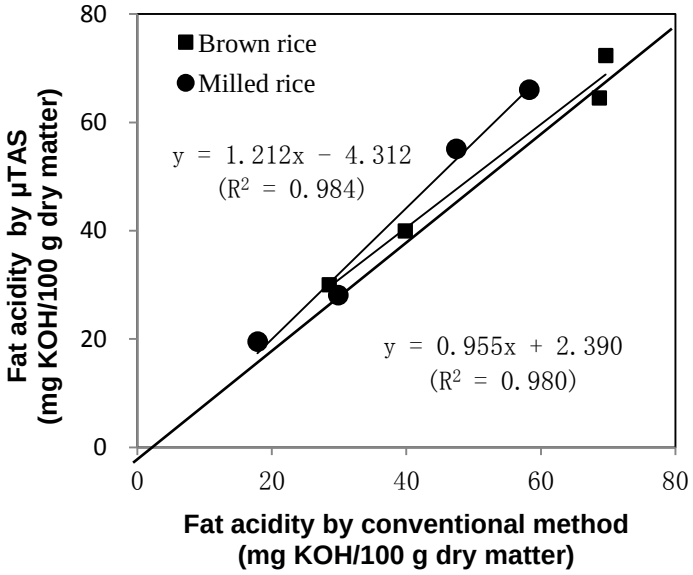

Fig. 3.4 Relationship between the fat acidity assessed by the conventional titration method and that assessed by µTAS.

3.4 Conclusions

A μTAS that can conduct on-chip acid–base titration was developed and used for rice freshness evaluation. Freshness could be distinguished between brown and milled rice samples, between samples harvested in different years, and between samples of different varieties. A good correlation was observed between the fat acidity assessed by the conventional titration method and that assessed using μTAS. Regression equations and coefficient of determinations of the two methods with brown and milled rice grains were as follows: $y = 0.955x + 2.390$ ($R^2 = 0.980$), $y = 1.212x - 4.312$ ($R^2 = 0.984$). Therefore, it is possible that rice grain freshness can be evaluated using μTAS. Compared with existing autometic titration device, which is expensive (approximately 650 thousand yen), large (around W (310 mm) × L (270 mm) × H (310 mm)) and time consuming, μTAS has smaller size (with a main working area of W (11 mm) × L (45 mm)) and was much cheaper and faster (approximately 2 minutes were required for one detection). The volume of one titration plug formed by automatic titration was hundreds times of that formed by μTAS depending on the burette capacity, less than 2 micro liter sample solution was needed for titration while automatic titration device usually need several milliliters. Nevertheless, for commercial use of μTAS in evaluating rice freshness, further improvements relating to the configulation and dimensions of μTAS are required.

CHAPTER 4

Development of μTAS for Pork Meat Freshness Evaluation

4.1 Introduction

As stated in chapter 1, pork is one of the most widely eaten meats in the world, accounting for about 38% of meat production worldwide. Figure 4.2 shows the production of pork in the whole world and Japan between 1967 and 2007, the production amount increased from 33.91 to 99.96 million tonnes in 40 years in the whole world, in Japan, the increment was 0.65 million tonnes. On the contrary, as shown in Figure 4.3, the consumption of pork meat increased continuously between 1967 and 2007 in the world. In Japan, the consumption of pork increased from 16.14 to 54.97 g $\cdot$capital^{-1} $\cdot$day^{-1}, which means that the consumption of pork increased more than three times in 40 years. According to the USDA's Foreign Agricultural Service, increasing of urbanization and disposable income has led to a rapid rise in pork consumption in China, where consumption of pork in 2006 was 20% higher than in 2002, and a further 5% increase projected in 2007.

With the increasing of production and consumption, pork is traded all over the world. 1.94 thousand tonnes of pork was imported in Japan in 2009, while 30 tones was exported, meanwhile, 4.36 million tonnes of pork was imported in the world in 2009, while 5.55 million tonnes was exported. In addition, pork freshness freshness decreases quickly during transportation as well as on shelf before sell, this is because, a large number of post-mortem reactions are initiated in pork (glycolysis, proteolysis and lipolysis) immediately after pig is slaughtered. Therefore, accurate evaluation of pork meat freshness is needed to minimize economic losses in the world transaction as well as to meet the requirement of consumers on pork quality.

Various methods have been studied based on the measurement of meat

post-mortem deteriorative changes associated with sensory quality, chemical changes and microbial growth (Park et al., 2000; Monroy et al., 2010; Gil et al., 2011). As I stated in chapter 1, sensory evaluation is a commonly used method for food freshness evaluation, it has also been widely used for meat freshness evaluation (Flores et al., 1999; Nitsch and Mudawi, 1999; Park et al., 2000). However, in most cases, it is subjective and inaccurate. Civera et al. (1999) studied the bacteriological changes of common cuttlefish, musky octopus, and broadtail squid coming from Ligurian Sea during storage in ice, the results showed that, total viable count appeared to be appropriate indicators to determine freshness level. Nevertheless, microbial methods always need long detection period and were hard for on-line detections (Blafsdbttir et al., 1997). Chemical methods measuring total volatile amines (Chen and Zhang, 2006), trimethylamine (Sengor et al., 2000) or pH change (Ohashi et al., 1991) have also been studied, however, these methods have difficulties in measuring early postmortem deterioration. Therefore, rapid and simple methods for meat freshness estimation have been required urgently.

As it is well known, ATP-related compounds present in the muscles of meat are degraded rapidly after death by the following process:

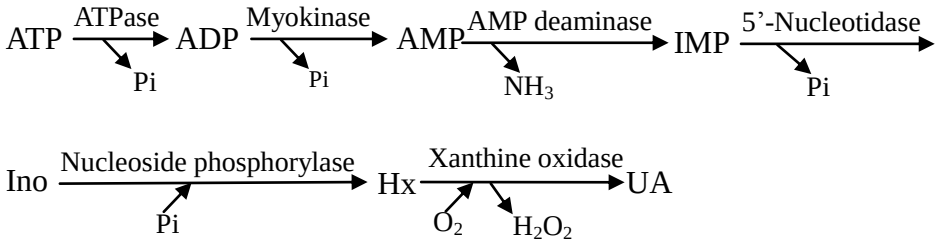

Fig. 4.1 Degradation of nucleotides presents in meat muscles.

Where ATP, ADP, AMP, IMP, Ino, Hx, and UA are adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, inosine monophosphate, inosine, hypoxanthine, and uric acid, respectively.

In the whole procedure, the degradation from ATP to IMP takes approximately 2

days after pig is slaughtered, the generated IMP is further degraded to Ino and Hx, which are accumulated during the later maturation process. Therefore, changes of the nucleotide and nucleoside metabolites resulting from ATP degradation were demonstrated as an important index for meat freshness evaluation, such as fish (Volpe and Mascini, 1996), chicken (Agui et al., 2006) and pork meat (Hernández-Cázares et al., 2010).

The researches of nucleotide to evaluate meat freshness is mainly based on K value (Vázquez-Ortiz et al., 1997; Kaminishi et al., 2000). K value is the ratio of Ino and Hx to the total amount of ATP-related compounds. The formula for K value is shown as following:

$$\text{K value}\ (\%) = \frac{\text{Ino+Hx}}{\text{ATP+ADP+AMP+IMP+Ino+Hx}} \times 100 \qquad \text{Eq. (4-1)}$$

HPLC method and biosensor are two main methods for calculation of K value (A. Mulchandani, 1990; Mulchandani et al., 1990; Fatih Ozogul, 2000; Ozogul et al., 2000; Etsuo Watanabe, 2005; Watanabe et al., 2005). However, as HPLC method needs large equipment and is hard for on-line detection, recently, more researches have done on calculation of K value by biosensors (Vázquez-Ortiz et al., 1997; Park et al., 2000). For calculation of K value, the amount of ATP-related compounds has to be calculated. Biosensors, which are based on determination of the produced H_2O_2 or the consumed O_2 during oxidation of Hx or its complexes through enzyme reactions, can be used to detect the amount of ATP-related compounds.

As I stated, ATP, ADP and AMP disappears rapidly at approximately 24 h after animal was slaughtered, moreover, the amount of IMP increases sharply at approximately 5-24 h after death and then gradually decreases, furthermore, the amount of Ino and Hx increases as the amount of IMP begins to decrease. Therefore, the freshness indicator K value has been simplified to the Ki value (Volpe and Mascini,

1996) and H value (Park et al., 2000), the formulas are shown in following equations (Eq. 4-2, 4-3).

$$\text{Ki value (\%)} = \frac{\text{Ino+Hx}}{\text{IMP+Ino+Hx}} \times 100 \qquad \text{Eq. (4-2)}$$

$$\text{H value (\%)} = \frac{\text{Hx}}{\text{IMP+Ino+Hx}} \times 100 \qquad \text{Eq. (4-3)}$$

Until now, biosensors have been widely studied for evaluation the freshness of fish meat, but rarely applied on pork meat evaluation; moreover, the studies of freshness indicators Ki value and H value for pork meat are limited. On the other hand, μTAS is an important enabling technology that uniquely integrates various research areas for a broad range of scientific and commercial applications. It offers novel approaches for solving crucial problems in the food industry as I stated in chapter 1. Therefore, in this research, a microchip fabricated with four sensors, has been developed for evaluation pork freshness according to K value, Ki value and H value. The most difficult and important part is the immobilization of the enzymes, the enzymes immobilized on working electrode with chitosan beads, glutaraldehyde, cellulose acetate (Okuma and Watanabe, 2002; Tkac et al., 2003; Hernández-Cázares et al., 2010) were reported. In our research, four immobilization methods were discussed.

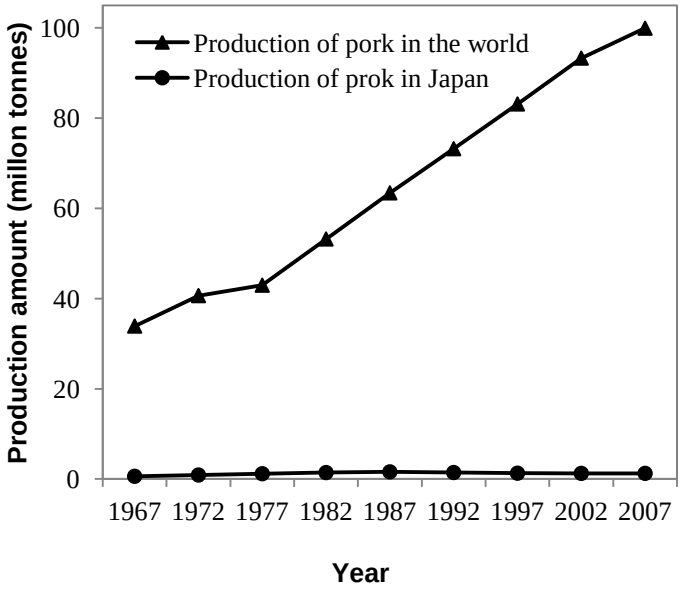

Fig. 4.2 Production of pork in Japan and the whole world in 1967-2007*.

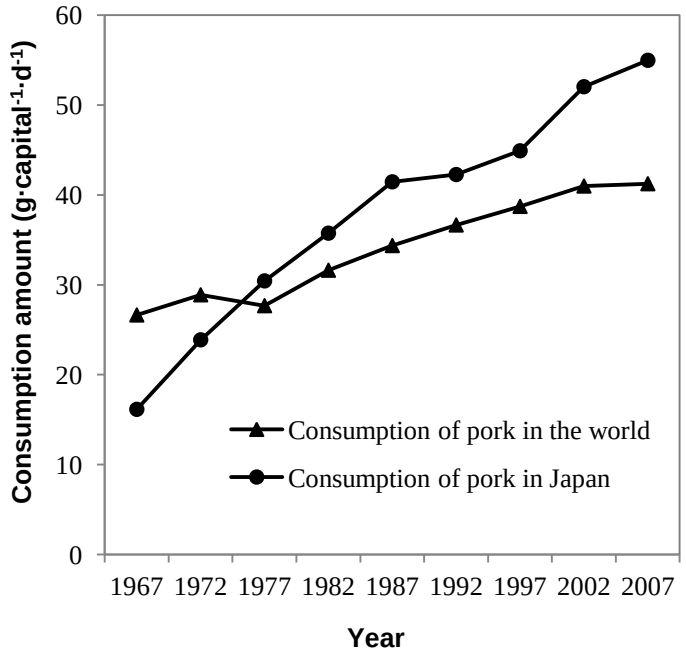

Fig. 4.3 Consumption of pork in Japan and the whole world in 1967-2007*.

* Source: FAOSTAT, http://faostat.fao.org/site/339/default.aspx

4.2 Materials and methods

4.2.1 Reagents and equipment

The following reagents and materials were obtained for the fabrication and evaluation of the devices: glass wafers (No. 7740, 3 in., 500-μm thick) from Corning Japan (Tokyo, Japan); a thick film photoresist, SU-8 from Microchem (Newton, MA, USA); precursor solution of polydimethylsiloxane (PDMS), KE-1300T from Shin-Etsu Chemical (Tokyo, Japan); potassium hydroxide, potassium dihydrogenphosphate, perchloric acid (70%), acetonitrile, potassium chloride and nucleotide standards (adenosine 5'-triphosphate, adenosine 5'-diphosphate, adenosine 5'-monophosphate, inosine 5'-monophosphate, inosine, and hypoxanthine) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Enzyme AP (alkaline phosphatase, EC 3.1.3.1 from calf intestinal mucosa) was purchased from Roche Applied Science (Indianapolis, USA); NP (nucleoside phosphorylase, bacterial EC 2.4.2.1) was purchased from Sigma-Aldrich Co. (St. Louis, MO, USA); XOD (xanthine oxidase, EC 1.1.3.22 from buttermilk) and AD (adenosine deaminase, EC 3.5.4.4 from Calf Spleen) were purchased from Wako Pure Chemical Industries, Ltd. Japan. Autolab PGSTAT12 potentiostat, (Eco Chemie, Utrecht, Netherlands). All other chemicals were of analytical-reagent grade and the water used for the research was obtained from a Millipore Milli-Q purification system.

4.2.2 Standards preparation

50 mM, pH 7.4 potassium dihydrogenphosphate solution containing 0.1 M KCl was used for preparing nucleotide standard solutions as well as the solution for blank test, pH was coordinated to 7.4 by 1 M NaOH using pH meter.

4.2.3 Detection principle

In this research, K value, Ki value, and H value have been calculated by the developed device through the detection the degradation of ATP-related compounds, the reaction procedure is shown in Figure 4.4:

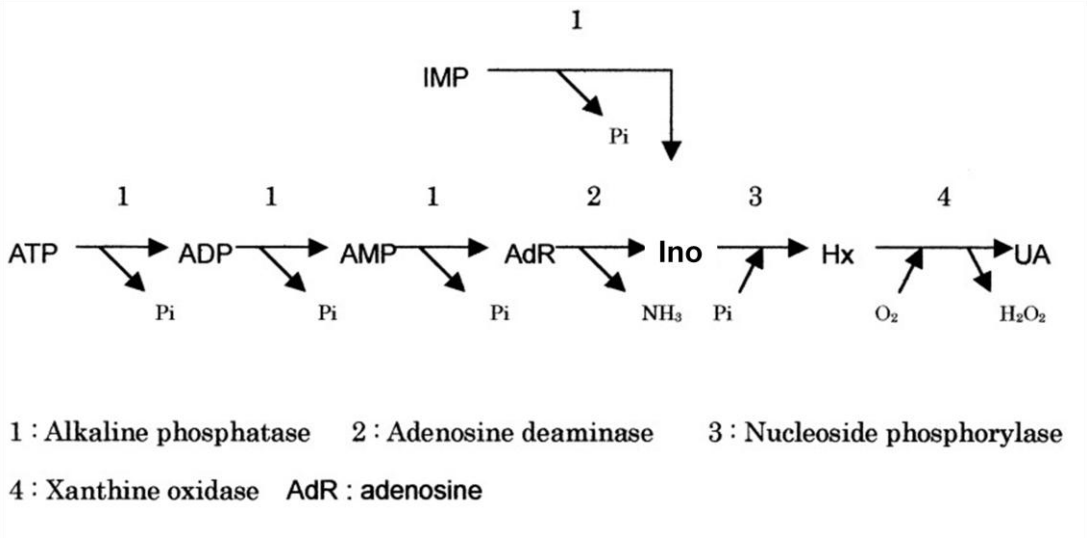

Fig. 4.4 Illustration of the detection procedure.

When AP, NP, AD, and XOD were used, all the ATP-related compounds were degraded to UA, the total amount of ATP-related compounds could be calculated. The total amount of IMP, Ino, and Hx could be obtained when AP, ZP, and XOD were used. Meanwhile, the total amount of Ino and Hx can be obtained by adding NP and XOD, while the amount of Hx could be obtained by adding XOD. Therefore, the denominators and numerators of K value, Ki value, and H value could be calculated. As Figure 4.5 shows, four sensors were fabricated on the developed device to implement the above four reactions. On sensor 4, AP, AD, NP, and XOD were used for degradation of ATP-related compounds, the total amount of the ATP-related compounds can be calculated from the produced H_2O_2 in the last step. Then, AP, NP, and XOD were used for sensor 3 to calculate the total amount of IMP, Ino and Hx. On sensor 2, NP and XOD were immobilized for detection the total of Ino and Hx. XOD was immobilized on the

working electrode of sensor 1 to calculate the amount of Hx. The production of H_2O_2 was recorded using the autolab PGSTAT12 potentiostat with GPES software, data was transferred from GPES to Excel for further process.

4.2.4 Fabrication of the device

As shown in Figure 4.5, the device was constructed with a sensor layer and a PDMS layer. On the W (14 mm) × L (32 mm) sensor layer, there are four groups of sensors. Each group contains a working electrode, a reference electrode, and an auxiliary electrode.

The electrode patterns were formed as follows: first, formation of chromium and platinum layer. Positive photoresist (S-1818) was spin coated on a clean glass wafer using a spin coater (1H-D7; Mikasa Corporation, Japan), after baking for 30 minutes at 80 ^{o}C， pattern of platinum electrode was formed on glass wafer under UV light on a mask aligner (MA-10; Mikasa, Japan), positive resist was used to remove the photoresist. The glass wafer was dried for a few minutes for chromium (5 min) and platinum (15min, 2 times) sputering (CFS-4ES-231; Shibaura Mecharonics Corporation, Japan). The substrate was immersed in acetone to remove the metal layers on the photoresist. This formed the patterns for the working and auxiliary electrodes. The platinum layer was also used as the base layer for the reference electrode. A silver layer was then formed only on the reference electrode area by the same lift-off process. A polyimide layer was formed to delineate the active areas for the electrodes and pad areas. To form the polyimide layer, a positive photoresist layer was patterned on the layer of polyimide (SP-341). During this process, the polyimide layer was also etched by the developer solution of the photoresist. The photoresist was then removed in ethanol. Finally, the polyimide patterns were cured at a final temperature of 285 ℃. The silver pattern was also covered with the polyimide layer except for two pinholes that were 88 μm in diameter. Silver chloride was grown from there by applying a current of

80 nA for 10 min in a 0.1 M KCl solution. The growth of the silver chloride layer and the potential were monitored simultaneously using a three-electrode configuration with a platinum plate auxiliary electrode and commercial Ag/AgCl reference electrode (2080A-06T, Horiba, Tokyo, Japan). The reaction chambers were formed by replica molding. A template was formed with the thick-film photoresist (SU-8), and the precursor solution of PDMS was poured onto the template. After PDMS was cured, it was removed from the template. Reaction chambers were thus obtained. Reaction solution was injected into the reaction chambers when PDMS layer was covered on the sensor layer, and connected with a autolab PGSTAT12 potentiostat to record the generation of H_2O_2 (Fig 4.5, 4.6).

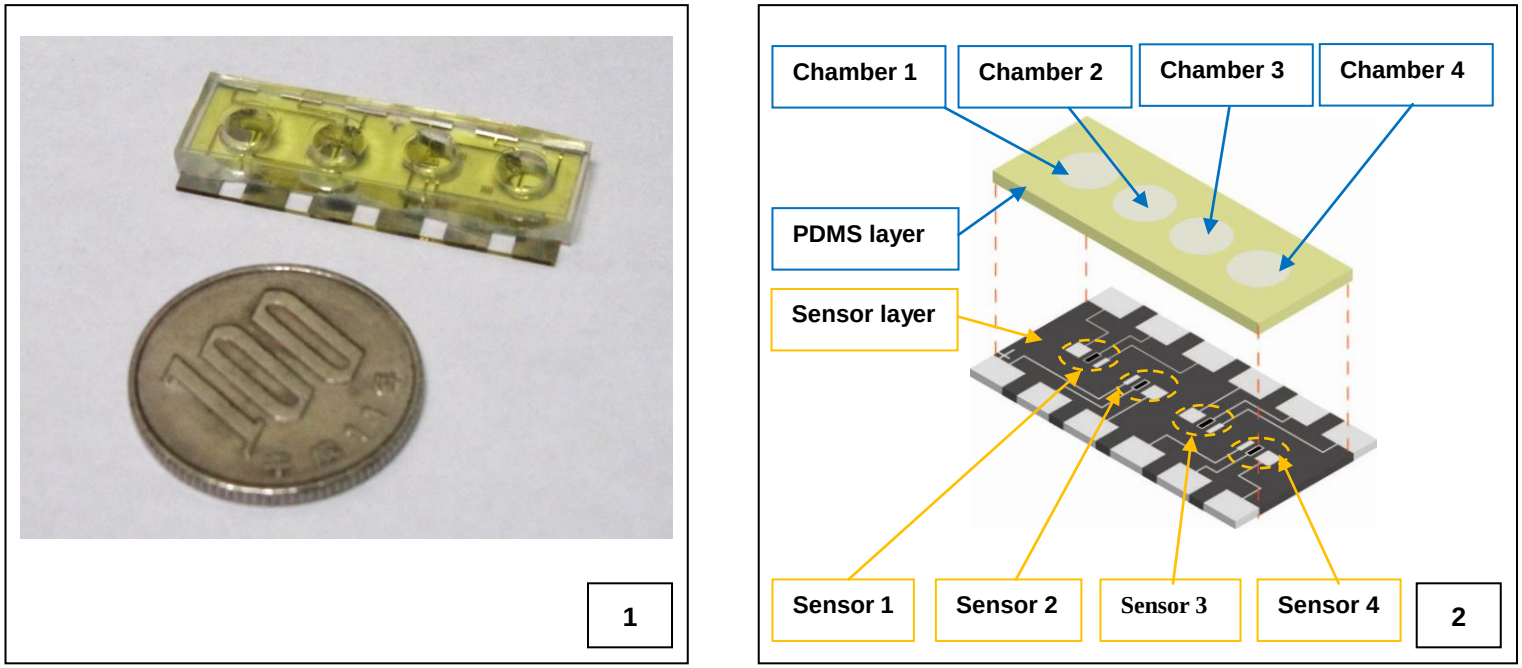

Fig. 4.5 Structure of the microdevice for pork meat freshness evaluation.

(1) Real size and the main parts of the device: PDMS with four reaction chambers and sensors.

(2) Components on the chip.

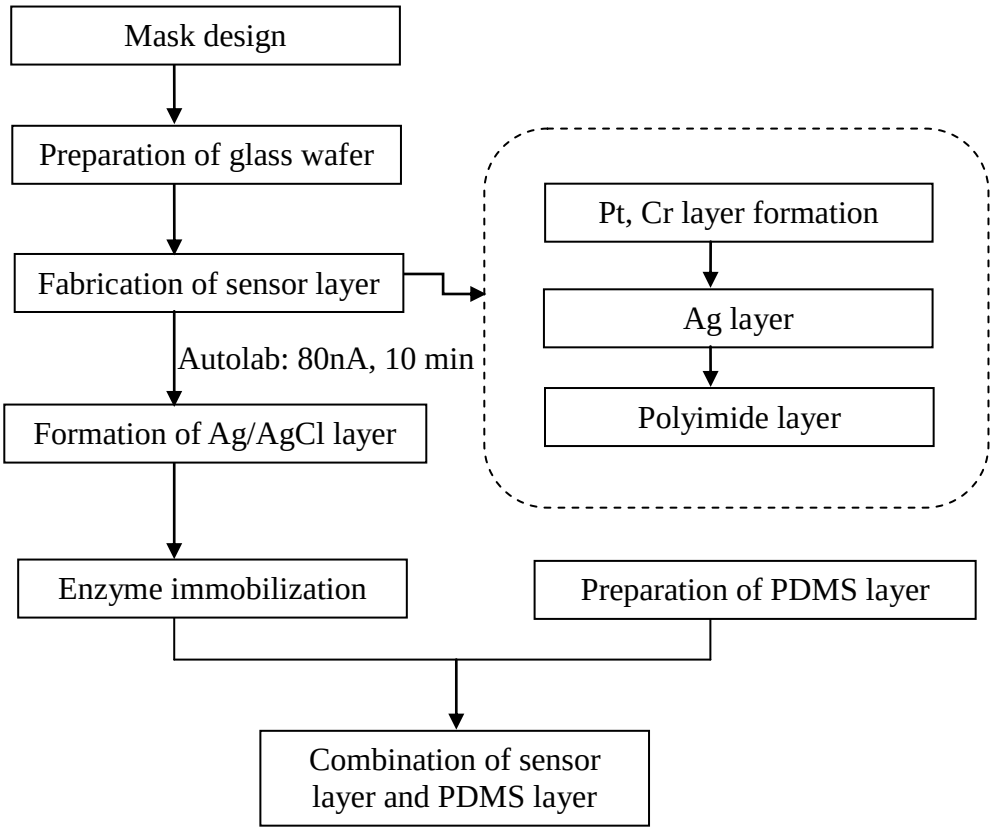

Fig. 4.6 Fabrication process for the μTAS.

4.2.5 Immobilization of the enzymes

Electrodes can be formed according to the procedures stated in 4.2.4; the next step is to immobilize enzymes on the surface of working electrodes. Based on the previous research, four immobilization methods were studied in this research. Sensors (chambers) were numbered as sensor (chamber) 1, 2, 3, and 4, the functional of each sensor was stated in 4.2.3.

4.2.5.1 Method 1: BSA, GA cross link method

BSA (albumin, from Bovine serum, Wako Pure Chemical Industries, Japan) and GA (glutaraldehyde solution, Wako Pure Chemical Industries, Japan) were widely used in sensors for immobilizing enzyme through cross link. In preliminary experiment, I noticed that, the volume and concentration of BSA and GA can affect the immobilization and enzyme activity, the enzyme layer was easy to remove during detection if concentration of GA was too low, the activity of enzyme decreased when the volume of BSA was too small or the concentration of GA was too large. Therefore, 0.1% (w/v) BSA, 0.2% (v/v), 0.5% (v/v) GA (0.2% (v/v) for sensor 1 and 2, 0.5% (v/v) for sensor 3 and 4) were used for immobilizing enzymes on working electrodes. The chip was stored at 4^{o}C for 8 hours after the enzyme layer dried at room temperature. Then, after immersing the chip in a glycine solution for 30 min, the fabricated device was covered with a PDMS layer for detection. As Figure 4.9 shows, a autolab PGSTAT12 potentiostat was connected with the three electrodes of the device to record the generation of H_2O_2 during the reaction, a potential of 0.7 V was applied during the detection. 20 μL, 50 mM, pH 7.4 potassium dihydrogenphosphate solution containing 0.1 M KCl was dropped into the reaction chambers for background detection, then, 20 μL standard solutions with ATP-related compounds were used to evaluate the device. 120 s was used as the response time for all the sensors.

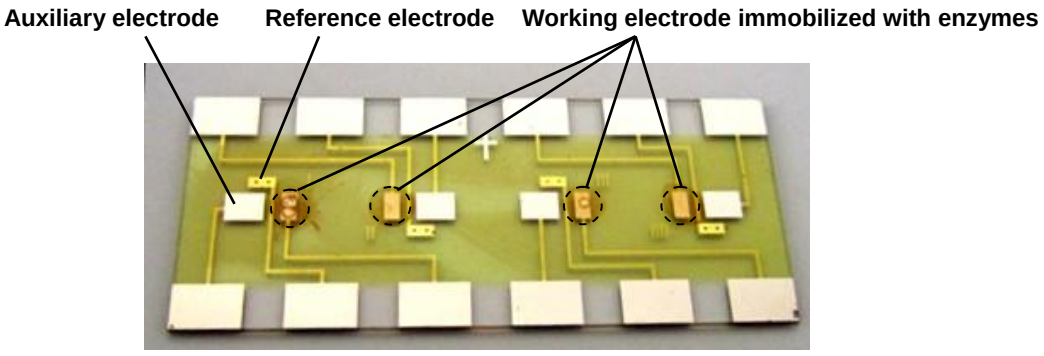

Fig. 4.7 Enzymes immobilization on the four sensors by method1.

4.2.5.2 Method 2: gel immobilized method

Photocurable polymer gel PVA-SbQ and SPP-H13 (Toyo Gosei, Japan), BSA, and GA were used as the second method for immobilizing enzymes. In preliminary experiment, I noticed that, when enzymes were immobilized by mixing 0.1% (w/v) BSA and 0.2% (v/v), 0.5% (v/v) GA (0.2% (v/v) for sensor 1 and 2; 0.5% (v/v) for sensor 3 and 4), gel, and enzyme directly, the immobilized enzyme layers on sensor 2, 3, and 4 were removed when the ship was immersed in a glycine solution. Therefore, for gel immobilization method, first, 0.1% (w/v) BSA, 0.2 % (v/v), 0.5% (v/v) GA (0.2% (v/v) for sensor 1 and 2, 0.5% (v/v) for sensor 3 and 4), and enzymes were immobilized on the working electrodes and waiting for 8 hours after the enzyme layers dried at room temperature. Then, after immersing the chip in a glycine solution for 30 min, the gel layers were formed on the enzyme layers under 365 nm UV light for 15 min. A PDMS layer containing four chambers, was covered with the fabricated sensor layer before detection. As Figure. 4.9 shows, autolab PGSTAT12 potentiostat was connected with the three electrodes of the device, 0.7 V potential was applied during detection. 20 μL, 50 mM, pH 7.4 KH_2PO_4 buffer solution was dropped into the reaction chambers for background detection, then, 20 μL standard solutions with ATP-related compounds were used to evaluate the device.

4.2.5.3 Method 3: CA immobilized method

CA (Cellulose acetate, Wako Pure Chemical Industries, Japan), ethanol (Wako Pure Chemical Industries, Japan), acetone (Wako Pure Chemical Industries, Japan), BSA, and GA were also used for immobilizing enzymes. When CA (dissolved by ethanol and acetone) was immobilized on the enzyme layer, which was immobilized by mixing enzymes, 0.2 % (v/v), 0.5 % (v/v) GA (0.2 % (v/v) for sensor 1 and 2, 0.5 % (v/v) for sensor 3 and 4), and 0.1% (w/v) BSA, and stored at 4^oC for 8 hours, the current

was easily saturated for the reaction in chamber 4. Concentration and volume ratio of ethanol and acetone can also affect the enzyme immobilization. Therefore, 1% CA (dissolved in ethanol/acetone: 1/1) and enzymes were dropped and mixed directly on the working electrode for immobilizing enzymes, and the enzyme layers were dried at room temperature for 3 hours. Then, a PDMS layer was covered with the sensor layer before detection. Figure 4.8 shows the immobilization surface of the device by method 2 and 3, for this method, because of the fast spread of the CA solution, the enzyme layer was thinner than method 2. As 4.2.5.1 stated, autolab PGSTAT12 potentiostat was connected with the three electrodes of the device, a potential of 0.7 V was applied during detection. 20 μL, 50 mM, pH 7.4 KH_2PO_4 buffer solution was dropped into the reaction chambers for background detection, then, 20 μL standard solutions with ATP-related compounds were used to evaluate the device. The response time was the same as the above method.

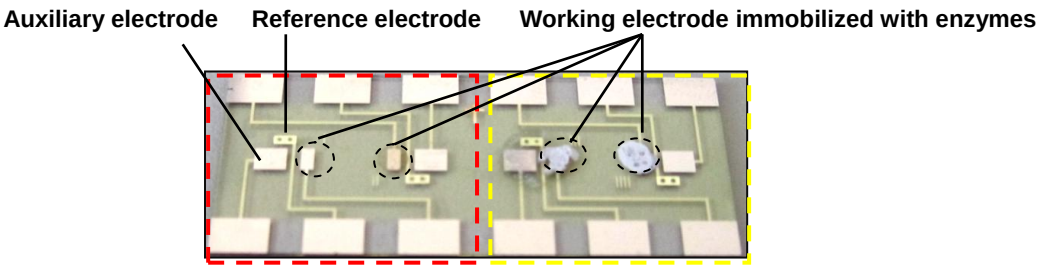

Fig. 4.8 Enzymes layers formed using method 2 and 3.

The left two sensors have enzymes immobilized by method 2; two sensors laying on the right have enzymes immobilized by method 3.

4.2.5.4 Method 4: immobilized and soluble enzyme combination method

Considering the affect of CA and gel on the thickness and activity of enzymes, method 1 was the best among the three methods. However, for chamber 4, four enzymes were immobilized simultaneously, therefore, after mixing, the activity of enzymes was lower than other chambers. On the other hand, the immobilized enzymes on sensor 3 and 4 were easy to lift during detection because of the limited amount of GA. To ensure the enzyme activity and stability, immobilized and soluble enzyme combination method was used. First, XOD was mixed with 0.1% (w/v) BSA and 0.2% (v/v) GA, 1.5 μL of the mixture was dropped on the working electrode of sensor 1 for immobilization. Then, XOD and NP were mixed with 0.1% (w/v) BSA and 0.2% (v/v) GA, then, 1.5 μL of the mixture was dropped on the working electrodes of sensor 2, 3, and 4, the device was stored at 4 ^{o}C for 8 hours before using it. After immersing the chip in a glycine solution for 30 min, the fabricated device was covered with a PDMS layer and connected to an autolab PGSTAT12 potentiostat, a potential of 0.7 V was applied during detection. 20 μL, 50 mM, pH 7.4 KH_2PO_4 buffer solution was dropped into the reaction chambers for background detection. During detection, sensor 1 and 2 can be used directly by adding 20 μL standard solutions; for sensor 3, 2 μL AP was dropped on the working electrode before adding standard solutions; for sensor 4, 2 μL AP and 1 μL AD were dropped on the working electrode before adding standard solutions.

The best condition for immobilizing XOD and NP was studied (Table 4-1) by changing the concentration of GA and the mixture ratio of BSA, GA, XOD, and NP. Moreover, the best volumes of AP and AD were also studied for sensor 3 and 4.

Table 4-1 The factors affected immobilization and current response.

Factors \ Conditions	1	2	3
GA (%)	0.1	0.2	0.5
BSA(0.1% (w/v)):GA(0.2% (v/v)): XO(μL)	2:1:1	1:1:2	ND
BSA(0.1% (w/v)):GA(0.2% (v/v)): XO: NP(μL)	1:1:1:1	0.5:0.5:2:1	ND
AP (μL)	1	2	ND
AD (μL)	1	2	ND

ND: means not detected

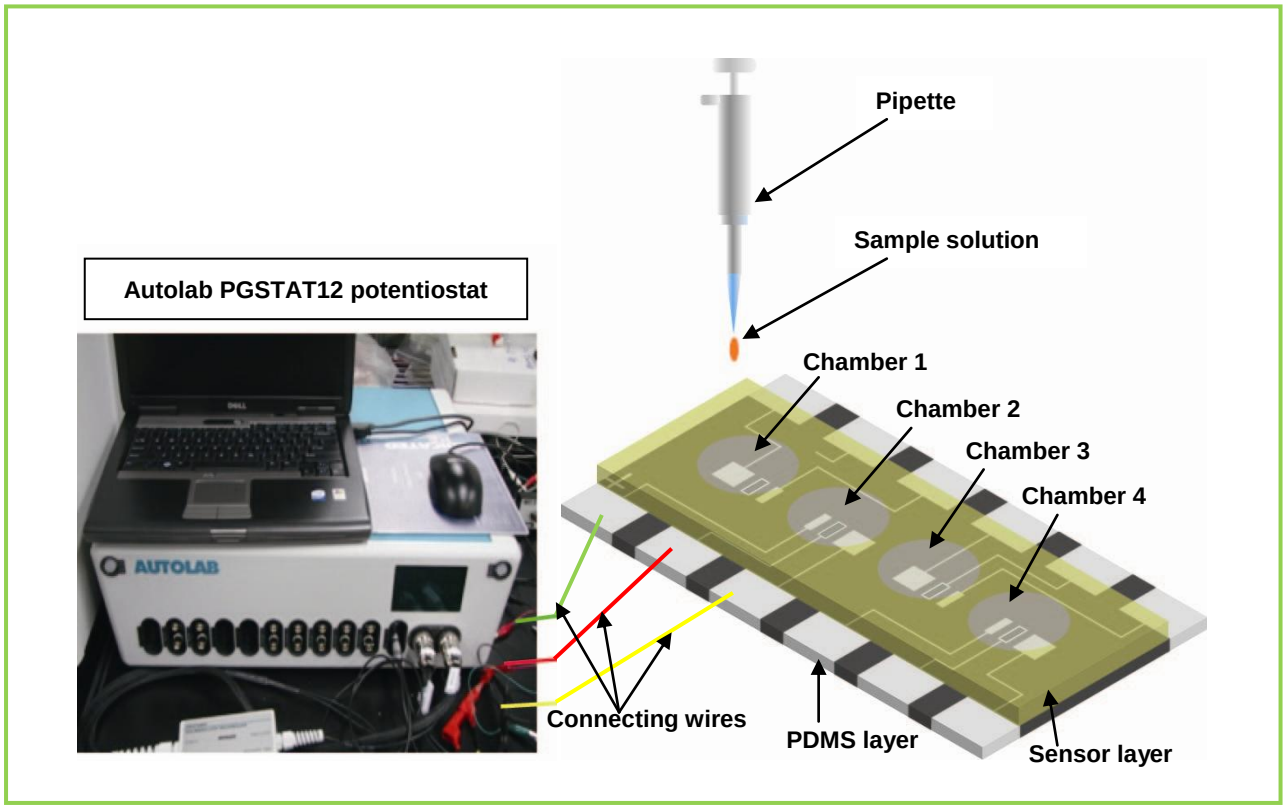

Fig 4.9 Sketch for the connection of microdevice and potentiostat.

4.3 Results and discussion

4.3.1 Comparison of enzyme immobilization methods

4.3.1.1 Method 1: BSA, GA cross link method

Table 4-2 shows the calibration equations and coefficients of determination for four chambers by immobilization method 1 (BSA, GA cross link method), the coefficients of determination are 0.887, 0.987, 0.987, and 0.986 for chamber 1 to 4 respectively. For chamber 1 and 2, the device can detect standard solution with the concentration up to 250 μM. For chamber 3 and 4, the current saturated at 150 μM and 100 μM, respectively. Moreover, when 20 μL, 250 μM standard solution was injected, the current generated in chamber 4 was smaller than chamber 3, this may because the activites of enzymes were not as good as chamber 3 because of the adding of AD or the reduction of BSA amount, in chamber 3, 2 μL BSA was used for immobilization while in chamber 4, only 1 μL BSA was used, BSA can help to stabilized the enzymes, if the amount of BSA is not enough, stability of enzyme may decreased after reusage for severe times.

4.3.1.2 Method 2: gel immobilized method

When gel was dropped on the surface of enzyme layer for immobilization, the stability of enzyme layer was better than using immobilization method 1, for which, the enzyme layer was removed after around 15 times detections. The calibration equations are shown in Table 4-3, the coefficients of determination are 0.829, 0.704, 0.839, and 0.923 for chamber 1 to 4 respectively, which were not as good as method 1. This may be caused by gel layer, although gel can help to improve the immobilization of enzymes, the activity of enzyme decreased when crapped by gel layer. From Figure 4.10, we can see that, the current saturated when the concentration was around 150 μM for chamber 2 to 4, for chamber 1, the increasing of the current was very limited, this may be affected

by a large amount of gel used for immobilization.

4.3.1.3 Method 3: CA immobilized method

CA has been widely used for enzyme immobilization (Bindra et al., 1991; Kurokawa et al., 1998; Mitchell, 2004). Table 4-4 shows the calibration equations and coefficients of determination of the four sensors by this method; the coefficients of determination are 0.738, 0.972, 0.996, and 0.931 for sensor 1 to 4 respectively. However, as Figure 4.11 shows, the produced current in chamber 4 was the lowest among the four chambers, this is because, when all the four enzymes mixed with CA solution, the concentration for each enzyme decreased compared with other chambers, for another, the spread of the CA solution was a little different among the four sensors, which also resulted in the bad repeatability of this method. Moreover, the linearity for sensor 1 was not as good as sensor 2 and 3, this should be connected, the activity of XOD decreased when enzymes were mixed with CA directly. Therefore, further improvement should be done if more than three enzymes are immobilized simultaneously.

4.3.1.4 Method 4: immobilized and soluble enzyme combination method

Considering the loss of enzyme activity in sensor 3 and 4 for all the above three methods, a new method was investigated. In preliminary experiment, we can see that this immobilized and soluble enzyme combination method can avoid the loss of enzyme activity during mixing.

The best immobilization condition for sensor 1 and 2 was investigated, as Figure 4. 12 shows, there is no big difference between the current generated by immobilizing enzymes with 0.1% (v/v) GA and 0.2% (v/v) GA. However, the current was very small and not steady when 0.5% (v/v) GA was used for immobilization. Therefore, 0.2% (v/v) GA was used for the future experiments. Furthermore, as different mixture ratios among BSA, GA and enzymes can affect the stability of the immobilized enzymes, Figure 4.13

shows the result for different ratios. When the ratio of BSA/GA/XOD = 1/1/2, the generated current was larger than the ratio BSA/GA/XOD = 2/1/1, which indicated that, increase of the enzyme amount can help to improve the value of generated current. On the other hand, on sensor 2, the ratio BSA/GA/XOD/NP = 0.5/0.5/2/1 was better than the ratio BSA/GA/XOD/NP = 1/1/1/1. Therefore, for sensor 1, the enzyme was immobilized with the ratio of BSA/GA/XOD = 1/1/2, while BSA/GA/XOD/NP = 0.5/0.5/2/1 was used for sensor 2, the immobilization condition for sensor 3 and 4 was the same as sensor 2. Furthermore, the investigation for the best volume of AD and AP showed that, the best volume for AD and AP was 1 μL and 2 μL respectively.

With the best immobilization condition, good calibration equations were obtained and shown in Table 4-5, the coefficients of determination are 0.887, 0.892, 0.924, and 0.913 for chamber 1 to 4, respectively.

Figure 4.15 shows the comparison of all the immobilization methods for each chamber. We can see that, the linearity for method 1 was good, nevertheless, the immobilization for sensor 3 and 4 was not good enough; for method 2, the current saturated when the concentration was around 150 μM for sensor 2 to 4, furthermore, the increase of the produced current was very limited on sensor 1; for method 3, the reproducibility of the device was not good because of the easily evaporation of the CA solution. As a result, method 4 was used as the best method for pork freshness evaluation.

Table 4-2 Calibration equations and coefficients of determination for chambers using immobilization method 1.

Chamber	Calibration equations	R^2
1	y = 0.559x - 9.561	0.887
2	y = 27.133x + 14.001	0.987
3	y = 34.160x + 30	0.987
4	y = 18x + 47	0.986

Table 4-3 Calibration equations and coefficients of determination for chambers using immobilization method 2.

Chamber	Calibration equations	R^2
1	y = 0.077x + 22.750	0.829
2	y = 27.467x + 121	0.704
3	y = 37.533x + 60.167	0.839
4	y = 109.930x + 3.667	0.923

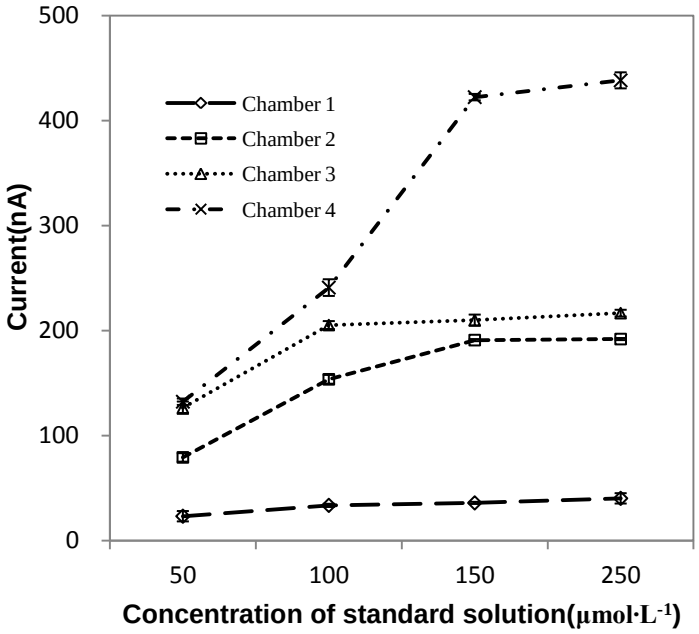

Fig. 4.10 Change of current with concentration in four chambers using immobilization method 2.

Labeled error bars indicate the standard deviation for each measurement (n = 3).

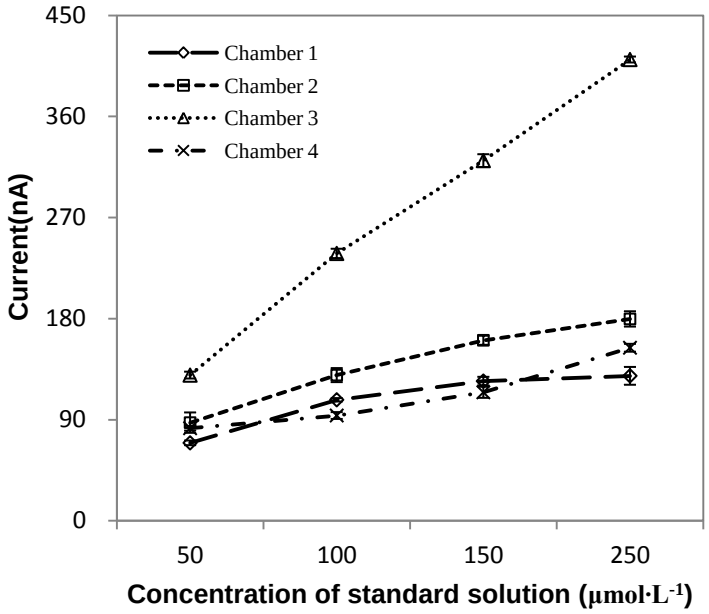

Fig. 4.11 Change of current with concentration in four chambers using immobilization method 3.

Labeled error bars indicate the standard deviation for each measurement (n = 3).

Table 4-4 Calibration equations and coefficients of determination for chambers using immobilization method 3.

Chamber	Calibration equations	R^2
1	y = 0.272x + 70.100	0.738
2	y = 30.800x + 62.332	0.972
3	y = 92.561x + 43.330	0.996
4	y = 23.567x + 52.167	0.931

Table 4-5 Calibration equations and coefficients of determination for chambers using immobilization method 4.

Chamber	Calibration equations	R^2
1	y = 0.679x - 12.350	0.887
2	y = 151.510x - 141.451	0.892
3	y = 144.100x - 33.992	0.924
4	y = 160.360x - 95.610	0.913

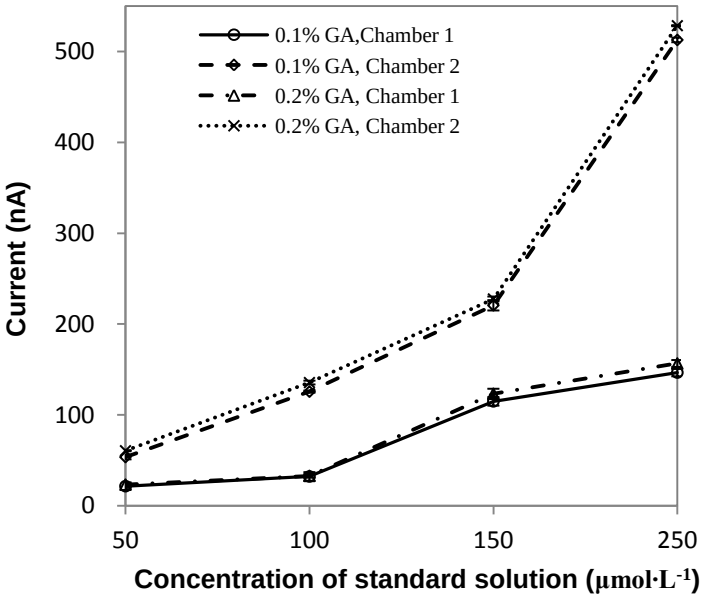

Fig. 4.12 The effect of GA concentration on enzyme immobilization for chamber 1 and 2.

Labeled error bars indicate the standard deviation for each measurement (n = 3).

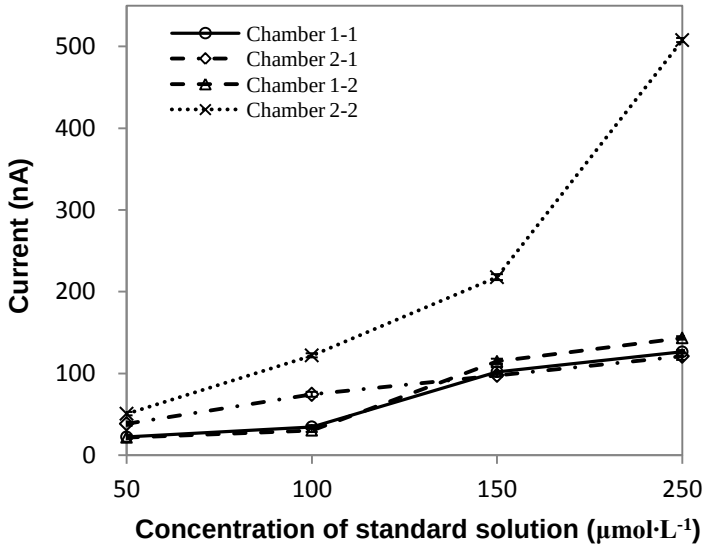

Fig. 4.13 The effect of ratios among BSA, GA, and enzymes on the immobilization for chamber 1 and 2.

Chamber1-1: the ratio of BSA:GA:XO is 2:1:1 in chamberv1; Chamber2-1: the ratio of BSA:GA:XO:NP is 1:1:1:1 in chamber 2; Chamber1-2: the ratio of BSA:GA:XO is 1:1:2 in chamber 1 ; Chamber2-2: the ratio of BSA:GA:XO:NP is 0.5:0.5:2:1 in chamber 2. Labeled error bars indicate the standard deviation for each measurement (n = 3).

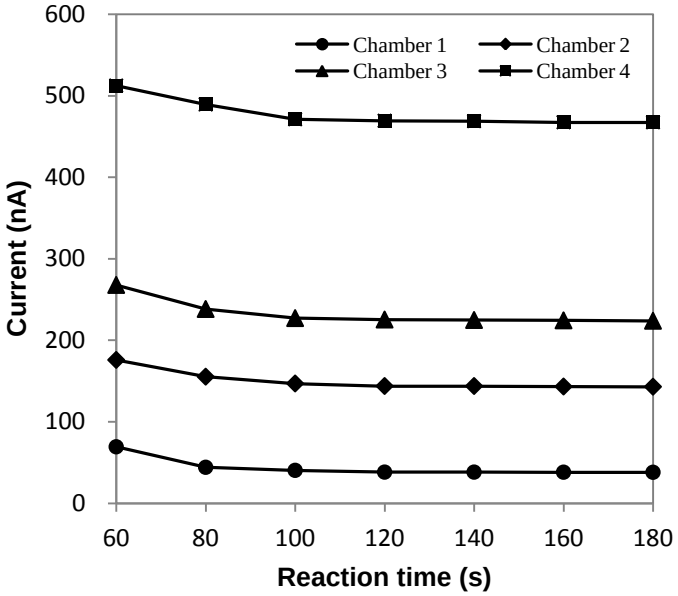

Fig. 4.14 The effect of reaction time on the sensor response.

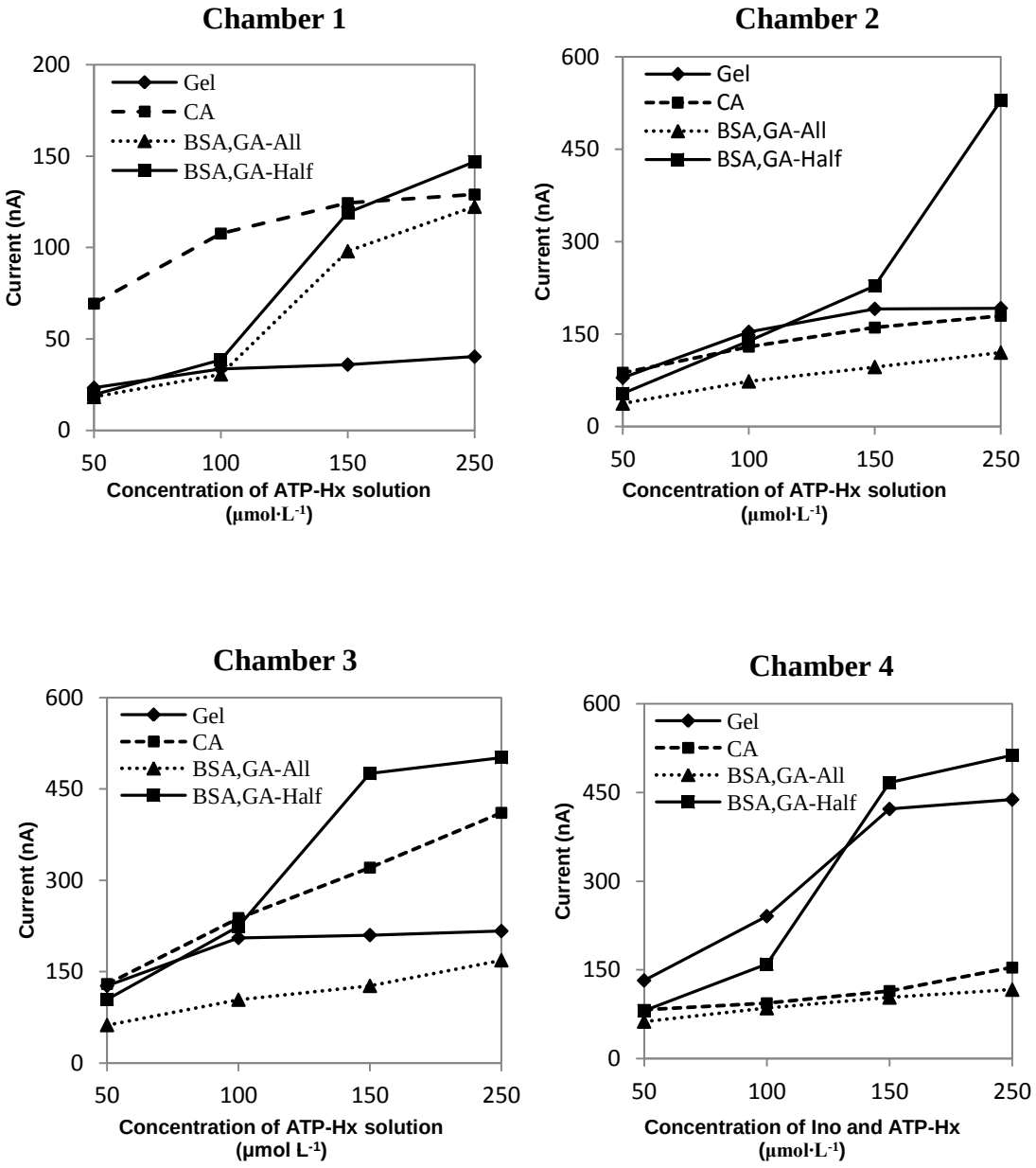

Fig. 4.15 Comparison of the four immobilization methods using standard solutions.

Gel:immobilized with gel, BSA and GA (method 2); CA: immobilized with CA (method 3); BSA, GA-All: immobilized with BSA and GA (method 1); BSA, GA-half:only XOD and NP were immobilized with BSA and GA (method4)

4.3.2 Stability and reproducibility of the device

Long-term stability and reproducibility of enzymes on sensor 1 and 2 immobilized according to method 4 were evaluated by measuring the current response at a fixed concentration of 100 μM and 50 μM, respectively. The stability was investigated over a period of three weeks, sensors were used every two days, and stored at 4^{o}C in two ways: one was stored with pH 7.4, 50 mM KH_2PO_4 buffer solution and the other stored without buffer solution. As Figure 4.16 shows, for sensor 2, the current response remained steady for one week, and the one stored in buffer solution decreased slower than that stored without buffer solution. The current decreased gradually from the beginning of the second week, and activity was exhausted when stored for three weeks in both of the two cases. On the other hand, for sensor 1, the current response kept steady for the first 11 days, and then decreased gradually, buffer solution can help to keep the enzyme stability on sensor 1, but the difference between these two store methods was not significant. Reproducibility of the device was shown in Table 4-6, the CV for all the other sensors was less than 10%.

Table 4-6 The reproducibility of repetitive detection of the device using method 4.

Chamber	1	2	3	4	5	Mean	STDev.	CV(%)
1	19.59	18.62	21.06	18.59	18.62	19.30	1.08	5.57
2	51.24	55.51	54.11	53.28	50.23	52.87	2.14	4.05
3	103.61	104.83	104.83	102.98	105.21	104.29	0.95	0.91
4	159.21	163.27	156.88	158.11	161.03	159.70	2.51	1.57

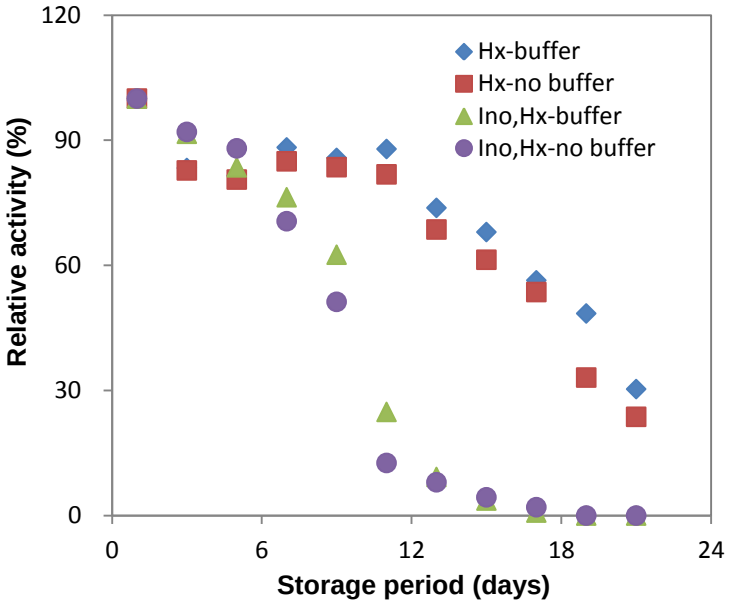

Fig 4.16 Long-term stability of the immobilized enzymes on sensor 1 and 2 stored at 4 ^{o}C with two store conditions for three weeks.

4.4 Conclusions

A novel μTAS for the determination of pork freshness based on K value has been developed. This device contains four reaction chambers and four sensors for calculation the numerators and denominators of K value, Ki value, and H value.

Four enzyme immobilization methods were studied: BSA and GA cross link method; gel immobilization; CA immobilization, and immobilized and soluble enzyme combination method. Good calibration equations have been obtained for all the methods. However, for method 1, the enzyme immobilization stability and reproducibility for sensor 3 and 4 were not good enough; for method 2, the current saturated when the concentration was around 150 μM for sensor 2 to 4, furthermore, the increase of the current was very limited on sensor 1; for method 3, the reproducibility of the device was not good because of the easily evaporation of the CA solution. Therefore, method 4 was decided as the best enzyme immobilization method for pork freshness evaluation.

The best immobilization conditions for sensor 1 and 2 were investigated. 0.2% (v/v) GA was the best concentration for enzyme immobilization for all the sensors. The best mix ratio for sensor 1 was BSA/GA/XO = 1/1/2, while ratio BSA/GA/XOD/NP = 0.5/0.5/2/1 was the optimum condition for sensor 2. The immobilization condition of NP and XOD on sensor 3 and 4 was the same as sensor 2, furthermore, the best amount of enzyme AP and AD was investigated. 2 μL AP was added to sensor 3 before detection; and 1 μL AD, 2 μl AP were added to sensor 4 before detection. With the best immobilization conditions, good calibration equations were obtained for all the sensors, and the coefficients of determination for sensor 1 to 4 are 0.887, 0.892, 0.924, and 0.913 respectively.

Long-term stability and reproducibility of the enzymes on the device immobilized according to method 4 were also studied, the enzymes were steady when stored at 4 ^{o}C for one week for sensor 2, and 11 days for sensor 1. Good reproducibility

of the device was observed (CV<10%).

CHAPTER 5

Evaluation of Pork Meat Freshness by μTAS and UPLC

5.1 Introduction

In chapter 4, an enzyme sensor based μTAS was developed and used for detection the standard solutions of ATP-related compounds, in this chapter, the freshness of pork was evaluated by the developed μTAS and UPLC equipment. As we known, the storage temperature can affect the degradation of ATP-related compounds, therefore, degradations of ATP-related compounds for pork stored at different temperatures as well as from different parts were also investigated by the developed μTAS. Finally, the correlation among K value, Ki value and H value was also investigated.

5.2 Materials and methods

5.2.1 Materials and reagents

Pork: samples of pork were obtained from JACC, Japan. Longissimus dorsi muscle (fifth and sixth thoracic vertebra) and left ham muscle (inner side) were used for this research. The samples were vacuum packaged using plastic bags and stored at 0 ^{o}C, 4 ^{o}C, and 6 ^{o}C. Samples were transferred to -80 ^{o}C at 1, 2, 4, 7, 14, 21 days until further analysis. Samples used for comparison the changes of ATP-related compounds in loin and ham were taken at 2, 4, 5, 6, 9 and 15 days, and moved to -80 ^{o}C until further analysis.

Reagents: all the reagents, standards and enzymes were the same as chapter 4.

5.2.2 Equipments

Autolab PGSTAT12 potentiostat (Eco Chemie, Utrecht, Netherlands) was used for record the changes of current during reaction. Ministirrer TR-100 (Pasolina, Japan) was

used for preparing the sample solution. pH meter, TPX-90Si (Tokyo Chemical Laboratories. Co. Ltd, Japan) was used to control the pH value of buffer solution and sample solution. Ultrasonic cleaning machine, US-105 (Esuenudi. Co. Ltd, Japan) was used to remove the bubbles in the mobile phase. High speed refrigerated centrifuge, 6800 (Kubota Company, Japan) was used to centrifuge the sample solution.

5.2.3 Pork preparation

Frozen tissue samples (5 g) were grounded and homogenized with 5% cold perchloric acid (25 mL) for 5 min. The extract was centrifuged at 3500 rpm for 10 min at 4 ^{o}C using high speed refrigerated centrifuge, and the supernatant was filtered through 0.45 μm filter paper and stored at 4 ^{o}C. Then, 15 mL, 5% cold perchloric acid was injected into the sediments again and stirred for 5 min. The extraction was centrifuged at 3500 rpm for 10 min at 4 ^{o}C, the supernatant was obtained through filtering, and was merged with solution obtained in first time. This solution was neutralized to pH 6.5 by adding potassium hydroxide and left to stand at 4 ^{o}C for 30 min. The supernatant solution was made up to 100 mL and then stored at -25 ^{o}C until analyzed. Meat extraction was thawed before injection, centrifuged according to the above method for 10 min and used for analysis by the developed μTAS and UPLC.

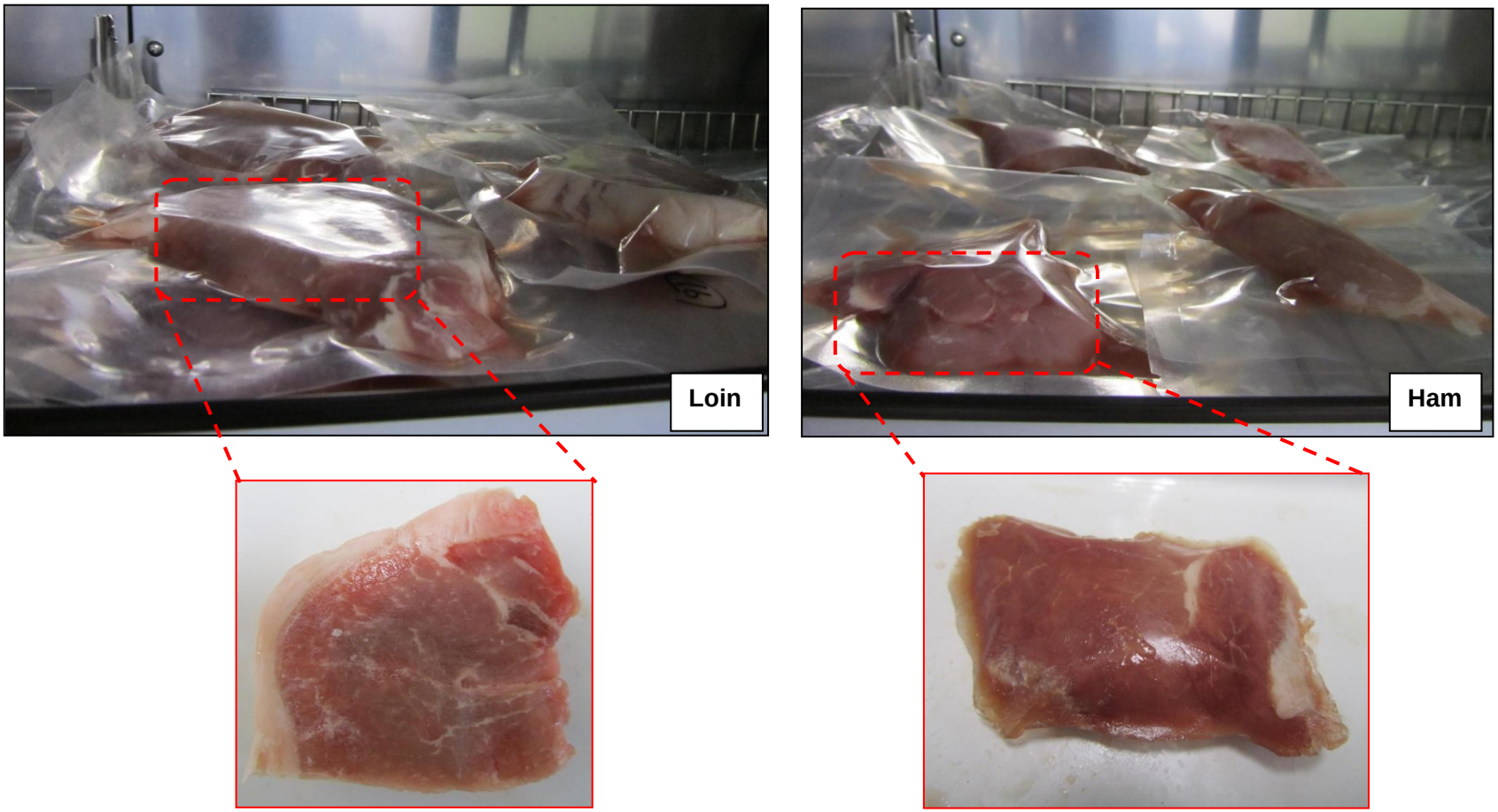

Fig. 5.1 Storage of loin and ham at 0 ^{o}C, 4 ^{o}C, and 6 ^{o}C.

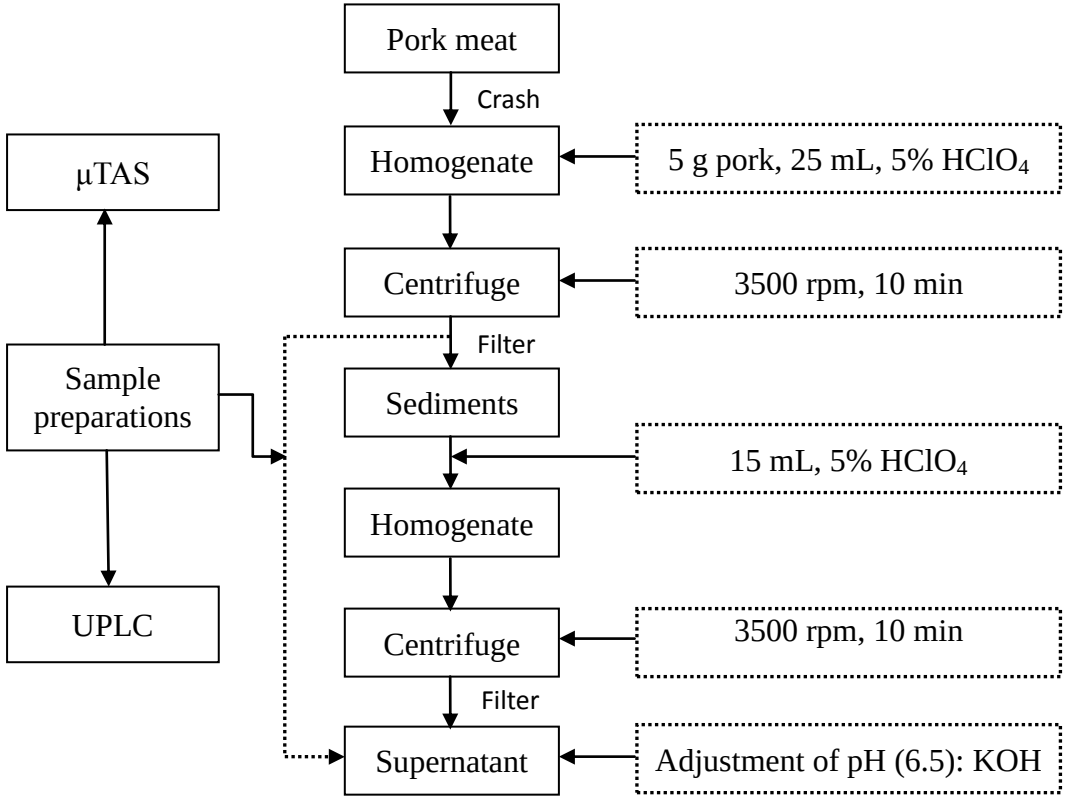

Fig. 5.2 Sample preparation for μTAS and UPLC.

5.2.4 Standards preparation

50 mM, pH 7.4 potassium dihydrogenphosphate solution was used for preparing nucleotides standard solutions of 6.5 μg mL^{-1}; 12.5 μg·mL^{-1}; 25 μg·mL^{-1}; 50 μg·mL^{-1}; 100 μg·mL^{-1}.

5.2.5 Description of the detection conditions of UPLC

The amount of ATP and its degradation products in pork samples was determined by a Waters Acquity UPLC (ultra performance liquid chromatography, Waters Corporation, USA) equipped with a PDA detector. A 5 μL of pork extraction was injected into the equipment. The mobile phase of 70/30 ACN/H_2O with 27 mM KH_2PO_4, pH 4.5 was used at a flow rate of 0.5 μL min^{-1}. Buffer solution was prepared daily and was filtered through 0.2 μm filter before detection. Separations were achieved on an Acquity UPLC BEH Amide column (2.1 x 100 mm, 1.7 μm) equilibrated at 25 ^{o}C. The detection was monitored at 260 nm. The separated compounds were identified by their respective retention times and spectrums. The quantification was performed by means of respective calibration curves.

5.2.6 Description of the detection procedures of μTAS

The calibration curves were obtained by the developed μTAS in chapter 4, in this chapter, the changes of ATP and its degradation compounds in pork meat as well as the value of freshness indicators, K value, Ki value and H value, were investigated. Loin muscle stored at 0 ^{o}C, 4 ^{o}C, and 6 ^{o}C was extracted and detected by the developed μTAS. Finally, pork samples taken from different parts (loin and inner side ham) were compared. The detection procedure by μTAS was the same as the detection of standard solutions described in chapter 4.

5.3 Results and discussion

5.3.1 Pork freshness evaluation by UPLC

5.3.1.1 Determination of the calibration curves for UPLC method

Six nucleotides have been well separated with the stated conditions, five different concentrations of standard solutions were used for establishing of the calibration equations for ATP, ADP, AMP, IMP, Ino, and Hx, as shown in Table 5-1, the coefficients of determination are 0.997, 0.996, 0.996, 0.996, 0.985, and 0.997 for ATP, ADP, AMP, IMP, Ino, and Hx respectively. Coefficient of variation was less than 1%.

5.3.1.2 Detection of pork freshness by UPLC method

The degradation of ATP, ADP, AMP, IMP, Ino, and Hx was investigated using loin stored at 4 ^{o}C for 15 days. ATP, ADP and AMP disappeared quickly after 2 days, the amount of IMP was the largest when meat stored for two days, the amount of IMP decreased gradually with the storage, while the amount of Ino and Hx increased. K value and Ki value were obtained for loin stored at 4 ^{o}C for 15 days, as Figure 5.5 shows, K value was smaller than Ki value during the storage, this is because, for Ki value, the denominator was only IMP, Ino, and Hx but six compounds were included in the denominator of K value. The amount of ATP, ADP, and AMP was very limited when stored for four days, the denominator for K value and Ki value becomes similar, therefore, Ki value can be used to replace of K value to evaluate pork freshness after four days storage.

Table 5-1 Calibration equations, coefficients of determination and CV of standard solution.

Compounds	Calibration equations	R^2	CV (%)*
Hx	y = 5113x + 3476	0.997	0.15
Ino	y = 4997x + 1800	0.996	0.32
IMP	y = 2451x + 4496	0.996	0.29
AMP	y = 3679x - 852	0.996	0.47
ADP	y = 1939x + 7279	0.985	0.31
ATP	y = 3092x + 12037	0.997	0.51

*CV: coefficient of variation (%).

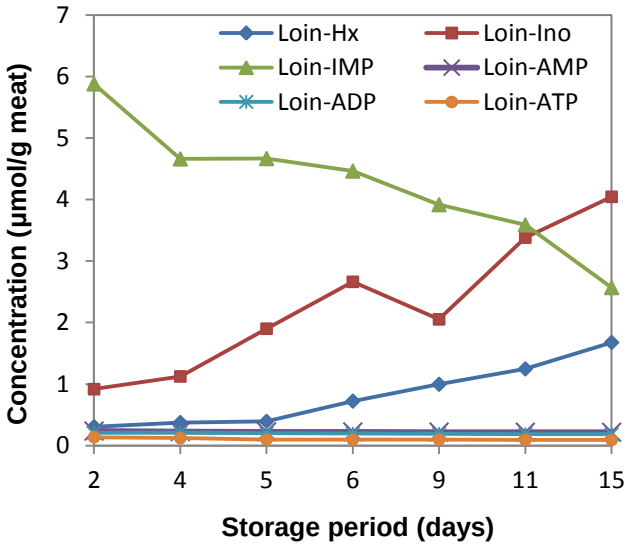

Fig. 5.3 Degradation of nucleotides in pork meat stored at 4 ^{o}C for 15 days.

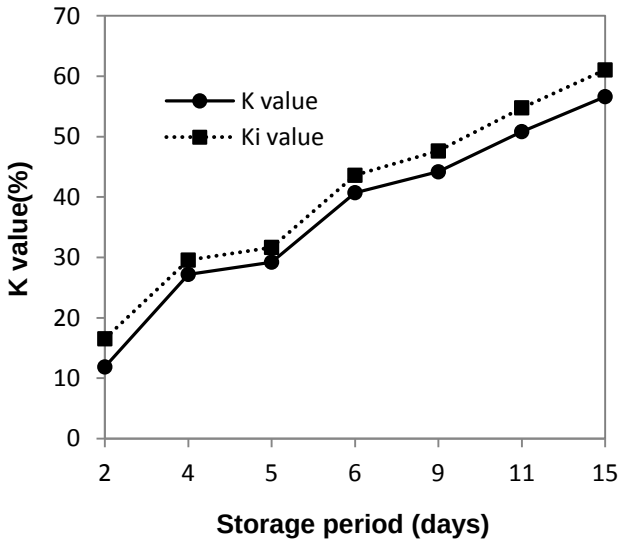

Fig. 5.4 Comparison of Kvalue and Ki value for pork meat stored at 4 ^{o}C for 15 days.

5.3.2 Pork freshness evaluation by μTAS

Figure 5.6 shows changes of K value for loin stored at 0 ^{o}C, 4 ^{o}C, and 6 ^{o}C for three weeks obtained by μTAS. For pork stored at 6 ^{o}C, K value was obviously larger than the others, while K value for loin stored at 0 ^{o}C and 4 ^{o}C was almost the same for first four days, therefore, it is hard to tell the differences for loin stored at 4 ^{o}C for less than 4 days by this device, but loin stored at 6 ^{o}C degraded faster than that stored at 0 ^{o}C and 4 ^{o}C. Figure 5.7 shows that the degradation of ATP-related compounds in loin was faster than ham when stored for 15 days, the result was accorded with UPLC method.

5.3.3 Corelationship between μTAS and UPLC

The correlations between the K value and Ki value determined by the developed μTAS and UPLC equipment were investigated. As Figure 5.5 shows, good agreement was observed between the two methods. The linear relationship had correlations of determination of 0.878 and 0.924, and the regression equations were: $y = 0.550x + 3.256$ and $y = 0.772x + 3.276$ for K value and Ki value respectively. Each assay could be performed within 2 min with the proposed sensor system, while the UPLC method equipment took 10 min. Moreover, correlations between K value and Ki value, K value and H value were investigated by the developed μTAS. As Figure 5.8 shows, good correlations (R^2=0.992) were observed between K value and Ki value, while for H value, the coefficient of determination was only 0.723. Therefore, Ki value can be used to replace K value for indicating the pork freshness level.

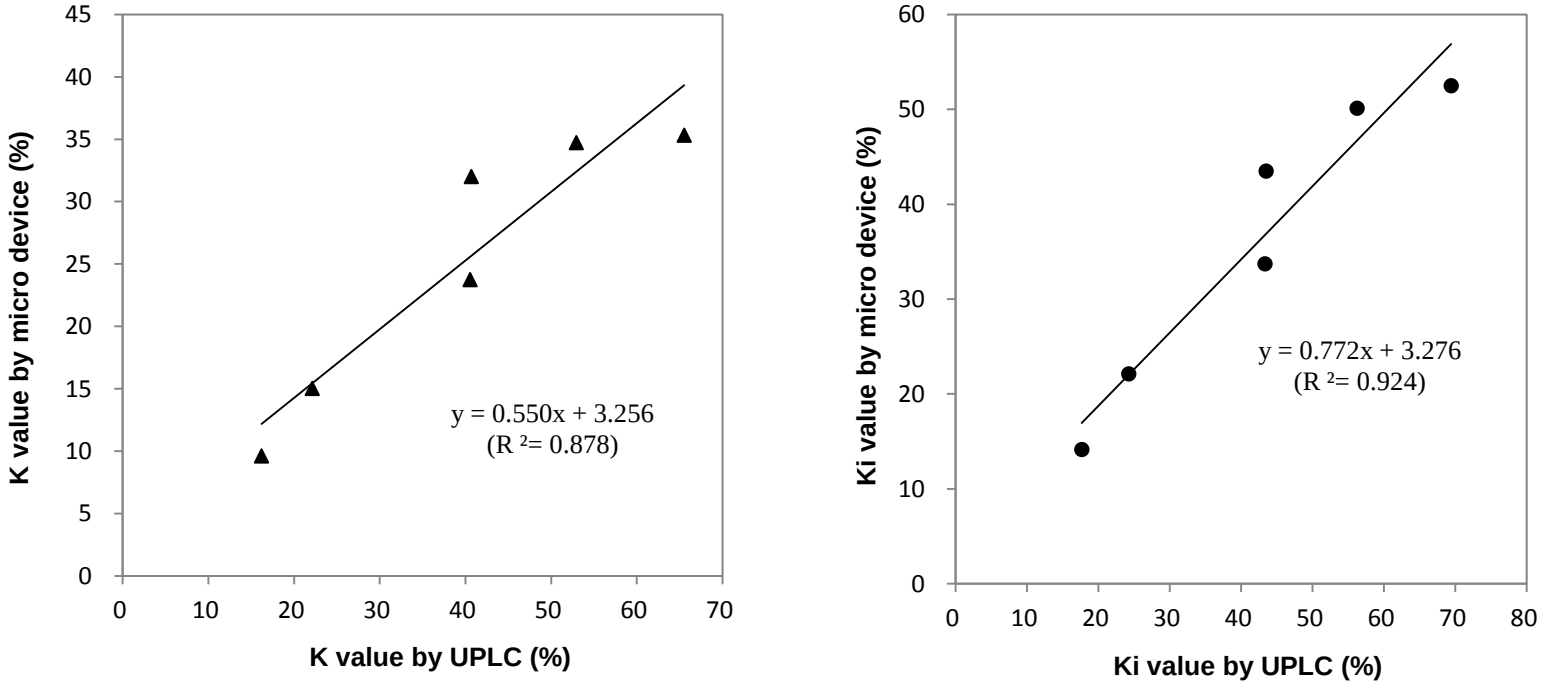

Fig. 5.5 Correlations of K value and Ki value measured by developed μTAS and UPLC in pork samples.

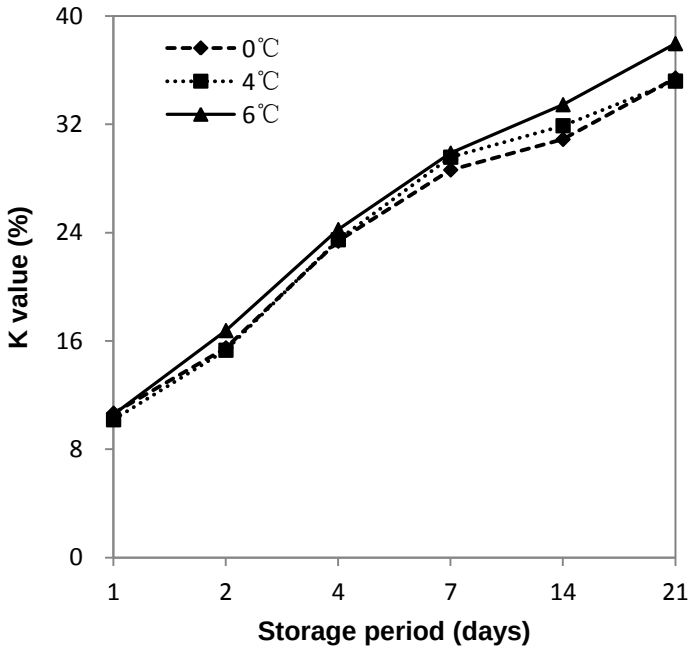

Fig. 5.6 Changes of K value for pork stored at different temperatures for three weeks.

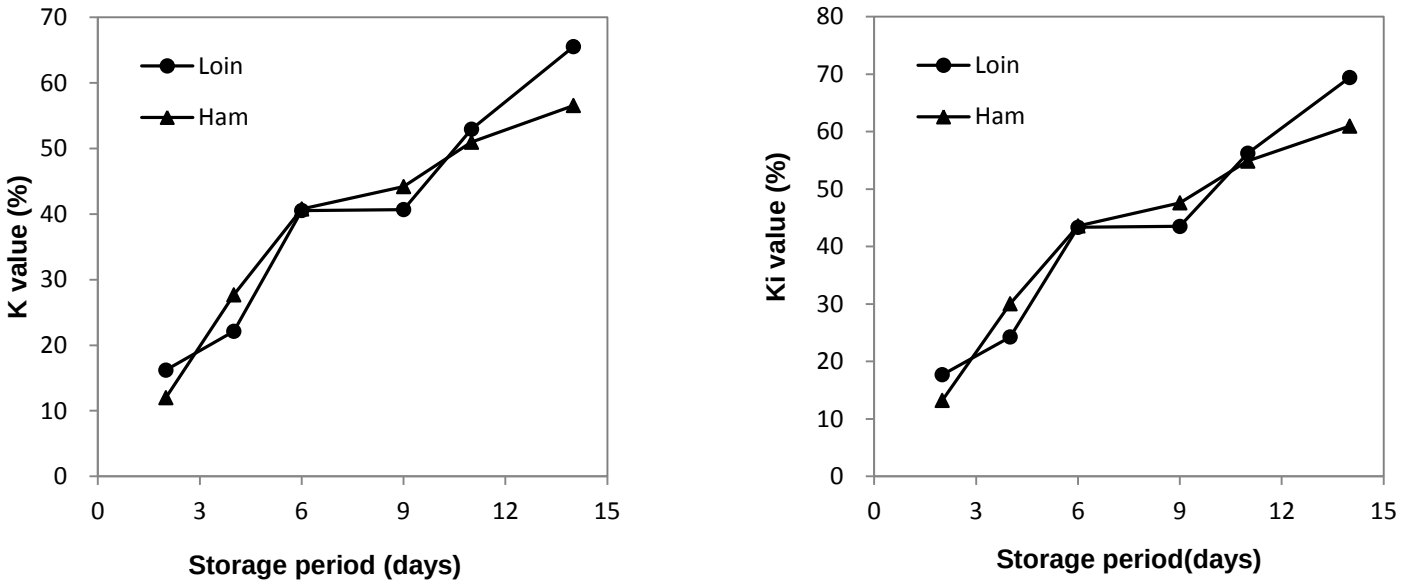

Fig. 5.7 Comparison of K value and Ki value for different parts of pork meat stored at 4 ^{o}C for three weeks.

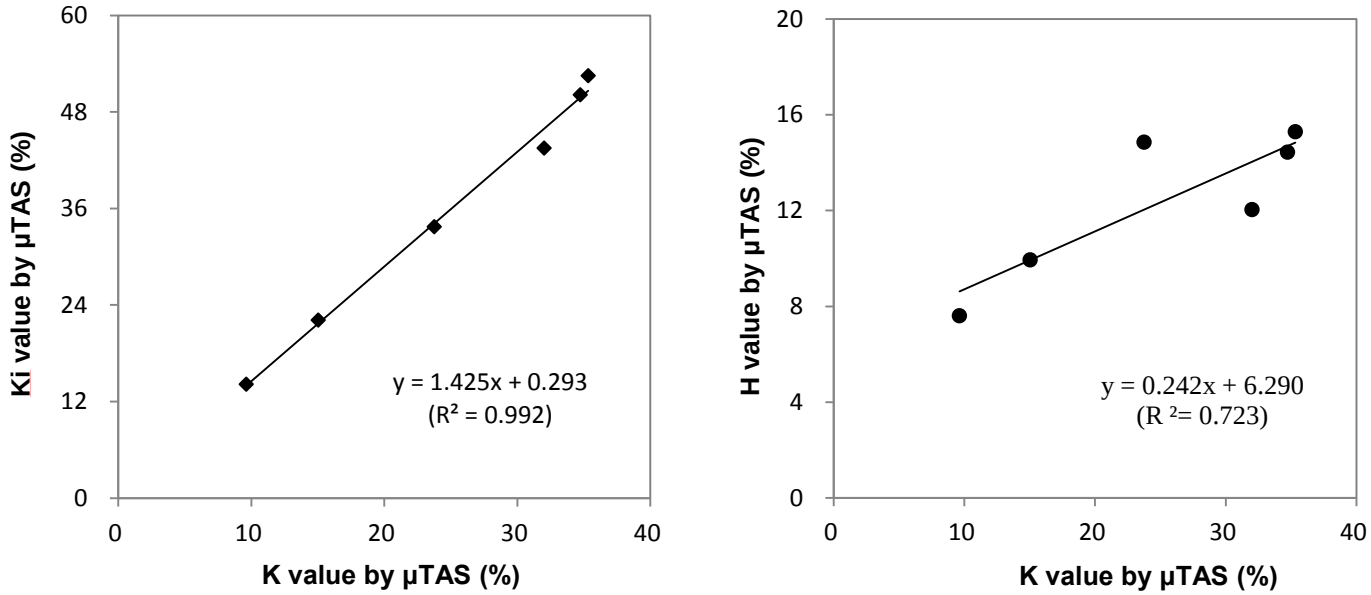

Fig 5.8 Correlations between K value and Ki value, K value and H value measured by the developed µTAS.

5.4 Conclusions

Freshness of pork was evaluated by UPLC euqipment. The retention time for separation all the ATP-related compounds was less than 5 min. Calibration equations were obtained for each compound for the later calculation. Coefficients of determination for ATP, ADP, AMP, IMP, Ino, and Hx are 0.997, 0.996, 0.996, 0.996, 0.985, and 0.997 respectively. Coefficient of variation was less than 1%.

The detection of pork meat by UPLC showed that, ATP, ADP, and AMP degraded quickly during 2 days, the amount of IMP was the largest when pork stored for two days, then decreased gradually with the storage, while the amount of Ino and Hx increased.

The developed μTAS was applied for detection the degradation of ATP-related compounds in pork stored at 0 ^{o}C, 4 ^{o}C, and 6 ^{o}C, the results showed that, the developed device could be used to tell pork stored at different temperatures. The investigation of loin and ham showed that, ATP-related compounds in loin degraded faster than ham when stored for 15 days, the result was accorded with UPLC method.

Investigation of the correlation of the K value and Ki value determined by the developed μTAS and UPLC showed that, good agreement was observed, the linear relationship had the coefficients of determination of 0.878 and 0.924 and the regression equations were $y = 0.556x + 3.256$ and $y = 0.772x + 3.276$ for K value and Ki value, respectively, the proposed device need only 2 min for each assay while the UPLC equipment took 10 min, therefore, the developed μTAS could be used to evaluate pork freshness, and it was faster and more convenient compared with UPLC equipment. Correlations between K value and Ki value as well as K value and H value were investigated by the developed device, good correlation (R^2=0.992) was obtained between K value and Ki value while for K value and H value, the coefficient of determination was only 0.723.

CHAPTER 6

Overall Conclusions

Foodstuff freshness plays an important role to daily life as well as to the world trade. The lost of freshness can not only affect the nutrition of food but also cause economic loss in the world trade. Moreover, some of the harmful substance generated with the aging of food, like the bacterials, aflatoxin and so on. The conventional methods for evaluation food freshness are either too expensive or need large equipment, therefore, in this study, I am trying to develop cheap and small-sized device for evaluation food freshness. μTAS (micro total analysis systems) is a system which can shrink a whole laboratory to a several centimeter chip, and is well known as small sample volumes, low consumption of reagents and portability. It has been widely used for clinical diagnostics, environmental concerns, immunoassays, proteins as well as DNA separation and analysis, but not for food freshness evaluation. In order to develop a small, cheap and easy device for food freshness evaluation, μTAS was developed to replace the conventional methods for rice and pork freshness evaluations.

This dissertation content could be drawn as follow:

1) In the first chapter, the general introduction of the importance, production and consumption of foodstuff were summarized. Moreover, this part also reviewed the former research in the areas of food freshness detection, the development of μTAS, the main technologies of μTAS, the application of it on food science as well as on other areas, especially the possibility to apply to food safety.

2) In the second chapter, a μTAS that can conduct on-chip acid–base titration was developed for rice freshness evaluation based on the conventional fat acidity method. The dimensions and shapes of the unit, as well as the length and width of the flow channels, were studied. Finally, the best size and shape was decided. The volume regulation cell in the optimum device can form plugs of uniform volume. The relative

standard deviation of the volume of the plugs formed in the volume regulation cell is much smaller than that of the fragments formed at the T-junction. The volume of the plug can be adjusted by changing the ratio of the flow rates of the titrant solution and air. Compared with existing autometic titration device, which is expensive (approximately 650 thousand yen), large (around W (310 mm) × L (270 mm) × H (310 mm)) and time consuming, the developed μTAS has smaller size (with a main working area of W (11 mm) × L (45 mm)) and was much cheaper and faster (approximately 2 minutes were required for one detection). Moreover, the volume of one titration plug formed by automatic titration was hundreds times of that formed by μTAS depending on the burette capacity.

3) In the third chapter, the developed μTAS was used for rice freshness evaluation, conventional fat acidity method was also used to verify the developed device. Therefore, Rice grains harvested in different years, with different species were studies by both conventional titration equipemtn and μTAS. The correlation between these two methods was also investigated with rice harvseted in different years. The resluts showed that, rice freshness could be distinguished between brown and milled rice samples, between samples harvested in different years, and between samples of different varieties. The same resluts for comparing frehsness of brown and milled rice freshness were stated by Tran et al. (2005) by conventional method, compared with this study, I have realized the same procedure on a faster and smaller device. A good correlation was observed between the fat acidity assessed by the conventional titration method and that assessed using μTAS. Regression equations and the coefficients of determination of the two methods with brown and milled rice grains were as follows: $y = 0.955x + 2.390$ ($R^2 = 0.980$), $y = 1.212x – 4.312$ ($R^2 = 0.984$). Therefore, it is possible that rice grain freshness can be evaluated using μTAS, for commercial use of μTAS in evaluating rice freshness.

4) Biosensors have been widely used for evaluation meat freshness, which were

more convenient than other methods, like HPLC, sensory evaluation, etc., considering the larger size of these sensors, some researchers have realized the fabrication of biosensors on μTAS, for example, μTAS for fish meat freshness evaluation has been studied. Therefore, in the fourth chapter, a μTAS fabricated with four sensors, was developed for evaluation pork freshness according to K value, Ki value and H value. This device contains four reaction chambers and four sensors to detect the denominators and numerators of K value, Ki value, and H value. Four immobilization methods were investigated: BSA and GA cross link method; gel immobilization; CA immobilization, as well as immobilized and soluble enzyme combination method. Good calibration equations have been obtained for all the methods. Considering the reproducibility of the device and the activity of the enzymes, method 4 was decided as the optimum method. The best immobilization conditions of the device were also studied, 0.2% (v/v) GA was the best concentration for immobilization for all the sensors. The best mixture ratio for sensor 1 was BSA/GA/XOD = 1/1/2, while GA/XOD/NP = 0.5/0.5/2/1 was used for sensor 2, the immobilization condition for sensor 3 and 4 was the same as sensor 2, furthermore, 2 μL AP was add for sensor 3 before detection; and 1 μL AD and 2 μL AP was add for sensor 4 before detection. With the best immobilization condition, the calibration equations were obtained using standard solution of nucleotides, and the coefficients of determination for sensor 1 to 4 are 0.887, 0.892, 0.924, and 0.913 respectively. Moreover, compared with the former studies, our device can evaluate meat freshness by K value, Ki value and H value simultaneously, and was smaller and more convenient.

5) In the fifth chapter, the freshness of pork meat was evaluated using developed μTAS and UPLC method. Pork meat stored at different temperatures as well as from different part was studied using the developed μTAS. The detection of pork meat using UPLC showed that, ATP, ADP, and AMP degraded quickly in two days, the amount of IMP was the largest when meat stored for two days, the amount of IMP decreased

gradually with the storage, while the amount of Ino and Hx increased. Ki value can be used to replace K value after four days storage to evaluate pork freshness. Coefficients of determination are 0.997, 0.996, 0.996, 0.996, 0.985, and 0.997 for ATP, ADP, AMP, IMP, Ino, and Hx, respectively.

μTAS could be used to detect pork meat stored at different temperatures. The investigation of loin and ham showed that, the degradation of ATP-related compounds in loin was faster than ham when stored for 15 days, the result was accorded with UPLC. Investigation of the correlation between the K and Ki value determined by the developed micordevice and by the UPLC method showed that, good agreement was observed, the linear relationship had coefficient of determinations of 0.878 and 0.924 and the regression equations were $y = 0.550x + 3.256$ and $y = 0.772x + 3.276$ for K and Ki value respectively, the proposed device need only 2 min for each assay while the UPLC took 10 min, the studies of HPLC and biosensors for evaluation meat freshness showed that, around 25 min and 5 min was needed for one assay respectively, therefore, this device was faster and more convenient compared with the former method, and can be used for pork freshness evaluation. Correlations between K value and Ki value as well as K value and H value were investigated using developed device, good correlation (R^2=0.992) was observed between K value and Ki value, while for H value, the coefficient of determination was only 0.723.

In this study, the research concerned with the development and application of μTAS for rice and pork meat freshness evaluation. It concluded that, the on-chip titration can be developed and used to detect the fat acidity in both brown and milled rice. The rice grains harvested in different years and with different varieties were detected using the developed device. Good correlation was obtained between the fat acidity by μTAS and the conventional method. Another sensor-base μTAS was developed, the different immobilization methods were discussed and finally the best immobilization condition for the four chambers was decided. The pork meat stored at

different temperatures were analyzed, changes of ATP-related compounds for loin and ham were discussed. The developed μTAS has a good correlation with UPLC method. The correlations of K value, Ki value and H value were also analyzed. In the future experiment, much research should be done to improve the developed devices. Firstly, for on-chip titration device, further research should be continue to improve the reuse times, in this study, the device couldn't be reused as the structure of inside wall of the device was broken after detection, secondly, for pork meat evaluation, further research should be done to improve the immobilization conditions. In this study, not all the enzymes were immobilized on the electrode, moreover, the results showed that, Ki value could be used to replace K value to show pork meat freshness, this was accorded with the former studies. Therefore, the number of the chambers on the device can be decreased to two for calculating of Ki value in the future.

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ACKNOWLEDGEMENTS

This dissertation would definitely not have been completed without the invaluable guidance, advice, support and encouragement of my academic devisor, Dr. Takaaki Satake, Professor of Graduate School of Life and Environmental Sciences, University of Tsukuba. I deeply appreciate for all the help from Professor Satake throughout my study in Japan.

I especially wish to express my thankfulness to Dr. Hiroaki Suzuki, Professor of Graduate School of Pure and Applied Sciences, University of Tsukuba, Japan, for his great advice and support for my experiment and paper written.

I deeply appreciate the suggestions and comments of Dr. Yutaka Kitamura, Dr. Toshiaki Nakajima and Dr. Shigeki Yoshida, Ass. Prof. of Graduate School of Life and Environmental Sciences, University of Tsukuba, Japan. I would also like to extend my thanks and appreciation to Doctor Kangquan Guo, Professor of College of Mechanical & Electronic Engineering, Northwest A&F University, China, for his support and encouragement.

I would also like to express my deep appreciation to Dr. Fumihiro Sassa, Mr. Daisuke Itoh, and Miss Eri Koyachi as well as all the other members of Professor Hiroaki Suzuki's laboratory for their advice and support.

I would also like to extend my thanks to all my lab members for their help during the experiment.

I also want to express my thanks to Zen-noh Central Research Institute for Feed and Livestock for their kindness to supply pork meat during my experiment.

Thanks to China Scholarship Council for providing me the chance to study and the financial support.

I also want to express my gratitude to my parents, my brother and my friends for their support and encouragement.