Paper

# Spectrophotometric Microdetermination of Urea in a Rice Wine by Using an Immobilized Acid Urease Column・FIA System

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Determination of urea in rice wines was performed by using a spectrophotometric flow-injection analysis (FIA) system introducing an acid urease column as a recognition element. An acid urease, having specific properties of showing catalytic activity in low pH range and tolerance to ethanol in comparison to those of a urease from jack-beans, was covalently immobilized onto porous glass beads and then, packed into a small polymer column. This flow-type of the biosensing system was assembled with a sample injection valve, the immobilized enzyme column, a gas-diffusion unit, and a flow-through quartz cell attached to a UV/VIS detector.

Standard urea solutions were measured through monitoring variations in absorbance resulting from pH shift due to ammonia molecules enzymatically generated. A wide, linear relationship was obtained between the concentration of urea (7.8 μM – 1.0 mM) and the change in absorbance. Followed by several investigations for application of this FIA system for measurement of urea in commercially available rice wines, the real samples were injected into the FIA system and urea in the samples were determined. These results were compared with those obtained with use of an F-kit method which was widely used for determination of urea. Comparative studies exhibited that this FIA system might be a powerful tool for urea determination in alcoholic beverages.

**Keywords** : Flow injection analysis, acid urease, urea, gas diffusion device, spectrophotometry

## 1. Introduction

Measurement of urea presents important information in many fields because urea is useful chemical as fertilizer[1],[2], feed[3],[4], marker for several diseases[5]~[7], cosmetic[8]~[10], and raw materials of synthetic resins[11],[12]. So, various kinds of samples such as foods, drinks, blood, urine, cosmetic, and so on were analyzed for determination of urea. Most of these investigations of the determination of urea have been based on the measurement of the changes in ammonia released from hydrolysis of urea catalyzed by urease. There are a great number of reports on flow injection analyses with electrochemical methods such as amperometry[13],[14], potentiometry[15],[16], conductometry[17],[18] and optical methods such as absorptiometry[19]~[21], fluorescence spectrophotometry[22]~[25], emission spectrophotometry[26]~[28] and calorimetry[29],[30]. All of these enzymatic methods for urea have used urease from jack bean, of which optimal pH is located in a narrow range of 7.0 – 8.0.

Rice wine is one of the most favorite alcoholic beverages in Japan, and it is drunk at cold and hot, or on the rocks and by cocktail, to say nothing of straight. Thus, rice wine is tasted in various styles. However, rice wine contains trace amounts of urea which was produced by yeasts during the fermentation of moromi mash[31]. Urea is considered as a precursor of ethylcarbamate[32], and the conversion to ethylcarbamate is promoted by heating under acid conditions. Formation of ethylcarbamate depends upon acidity, temperature, preservation period, amount of ethyl alcohol and the concentration of urea[31]. Since ethylcarbamate has been known to be carcinogenic, teratogenic, and mutagenic[33],[34], removal of urea being a precursor of ethylcarbamate from rice wine is very important task to be solved urgently[32], and the application of acid urease to remove urea[31],[35] or use of a genetically engineered sake-yeast producing no urea[36] has been tried. Thus, the monitoring of urea in rice wine is required. In the case of determination of urea in alcohol beverages like rice wines, acid urease from *Lactoballilus fermentum* is preferable against that of jack bean, because acid urease can catalyze the urea even in lower pH region and also has tolerance of alcohol. Therefore, we had developed a calorimetric determination system for urea with use of an acid urease as a recognition element[37]~[39]. However, this calorimetric system was not always sufficiently sensitive to measure urea in rice wine. In previous studies[40]~[42], we reported a monitoring system for measurement of urea with use of an acid urease column in a FIA system based on a photometrical detection. In this study, we would like to report the properties and application of the system to microdetermination of urea in real samples.

## 2. Experimental

**2.1 Materials and Reagents**　Acid urease (from *Lactobacillus fermentum*) containing 95 % lactose, NAGAPSHIN, was kindly provided by Nagase & Co., Ltd. (Osaka, Japan). Controlled-Pore glass (CPG, mean pore diameter 24.2 nm, particle size 120 – 200 mesh) was purchased from Funakoshi Co., Ltd. (Tokyo). A gas diffusion device and porous PTFE (polytetrafluoroethylene) tubing (mean pore diameter 1.0 μm, inner diameter: 1 mm, outer diameter: 2 mm, length of part of gas

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diffusion layer: 120 mm) were purchased from F·I·A Instruments Co., Ltd. (Tokyo) and Flon Industry (Tokyo), respectively. An F-kit (TC Ammonia) was purchased from Nippon Beehringer Ingelheim Co., Ltd. (Tokyo). Ion exchange resins (Amberlite MB-2, pore diameter; 300 - 850 μm) were obtained from Organo Corporation (Tokyo). A filter for injection sample, Millex®-GV, was purchased from Millipore, Tokyo. Urea (biochemical grade) was from Wako Pure Chemical Industies, Ltd (Osaka, Japan). All other reagents were commercially available and of analytical grade. Ultrapure water with a resistivity of 18.2 MΩ–cm was obtained from an EQG-3S system (Nippon Millipore K. K., Tokyo), and used in all procedures.

**2.2 Purification of Acid Urease from the Bulk Enzyme**

NAGAPSHIN (25 g) was dissolved in 500 ml phosphate buffer (50 mM, pH 7.0) and was filtered with Microfilter (AstroPore disc capsule type CACL 45 2.5 CMD, pore size: 5.0 μm; Fuji Photo Film Co. LTD, Tokyo) to remove insoluble matters. The filtrate was concentrated down to 50 ml with an ultrafiltration method (Vivaflow 50: Mw 100,000, Sartorius, Tokyo). After the concentrated solution was diluted to 300 ml with 5.0 mM phosphate buffer, the diluent was desalted down to 50 ml by using Vivaflow 50. This desalting process was repeated twice. The desalted sample was lyophilized (FREEZE DRYER FDU-830, EYELA) to obtain acid urease powder.

**2.3 Immobilization of Acid Urease** Acid urease was covalently immobilized onto alkylaminated CPG as described previously[43]. The glass beads (3.3 g) was boiled in nitric acid (5 %, 150 ml) for 45 min on hot plate. After allowed to stand at a room temperature, the beads were recovered with a glass filter and washed with pure water (2 l) and then dried in an oven at 95 ℃. The washed beads were alkylaminated with 10 % γ-APTES (γ-aminopropyltriethoxysilane; pH 3.45) in a shaking water-bath at 75 ℃ for 3 hours. Then, the glass beads were collected by filtration and washed with pure water (20 x 3 ml), and the beads were dried in an oven (115 ℃, over night). The alkylamninated glass beads (1.0 g) were activated with glutaraldehyde (2.5 %, 25 ml) under reduced pressure, and then, acid urease solution (120 mg/ml, 9.0 ml) prepared in phosphate buffer (50 mM, pH 7.0) was coupled with the glass beads with shaking at 4 ℃ for 50 hours. Finally, the immobilized preparations were treated with 10 % sodium borohydrate solution that was prepared by mixing with 0.2 M phosphate buffer (pH 5.0, 5.0 ml), 0.1 M $NaH_2PO_4$ (1.0 ml) and 4.0 ml pure water. The reducing processs was repeated ten times and then washed with phosphate buffer (0.1 M, pH 7.0). The yield was calculated by measuring absorbance at 280 nm in the enzyme solutions before and after the coupling process. The immobilization yield was 89 %.

**2.4 Flow System and Procedure** A schematic diagram of the flow system is shown in Fig. 1. The system was assembled with two double-plunger pumps (Sanuki DM3M-2044, DMX-2000, Sanuki Industry Co., Ltd., Tokyo), a rotary injection valve with a 100 μl sample loop, the immobilized acid urease column (300 μl) with a water-jacket, a gas-diffusion unit, a UV/VIS detector (UV-970, JASCO Corp., Tokyo) with a quartz flow-through cell (volume 32 μl, light-path length 10 mm), and a pen recorder (Multi-Pen Recorder; type R-62M3, Rikadenki Kogyo Co. Ltd., Tokyo). The temperature around the gas diffusion unit was regulated with a constant temperature bath (F·I·A Instruments Co., Ltd.).

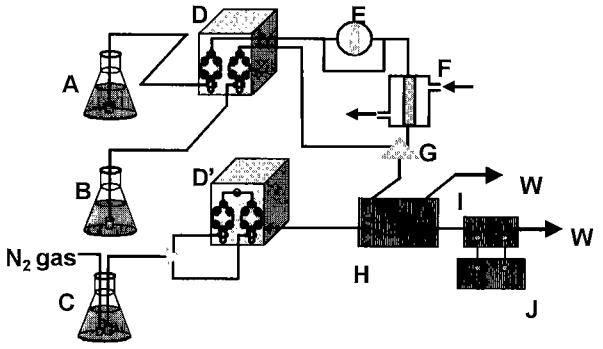


Fig. 1. Schematic diagram of a photometric flow-injection system for the determination of urea using an immobilized acid urease column. A=carrier (50 mM citrate buffer, pH 5.0, 30 ℃, 0.2ml $min^{-1}$) ; B=alkaline buffer for gas-diffusion (0.1 M sodium phosphate, pH 12.0, 30 ℃, 0.2ml $min^{-1}$) ; C=coloring reagent (0.15 mM Thymol Blue, pH 8.4, 0.4 ml $min^{-1}$) ; D,D'=double plunger pump ; E=rotary injection valve with a sample loop ; F=immobilized acid urease column housed in a water-jacketed holder (30 ℃) ; G=three-way mixing joint ; H=gas-diffusion unit ; I=UV/VIS detector ; J=pen recorder ; W=waste.

Citrate buffer (50 mM, pH 5.0) as the carrier solution (0.4 ml $min^{-1}$) was successively pumped through the system. Sample solutions were introduced into the system via the rotary injection valve. Ammonium ions formed in the enzymatic hydrolysis of urea were converted to gaseous ammonia molecules by mixing with the strongly alkaline buffer (gas-diffusion buffer: 100 mM sodium phosphate, pH 12.0), and the mixed solution (0.8 ml $min^{-1}$) was transferred to the gas-diffusion unit consisting of a double tubing structure[44),(45)]. The absorbance of Thymol Blue flowing streams in the PTFE tubing was varied by gaseous ammonia diffusion across the PTFE tubing, and subsequent increase in absorbance at 596 nm attributable to the reaction was successively monitored by a flow-through type of a UV/VIS detector and displayed on the pen recorder. The coloring reagent solution (Thymol Blue solution, 0.15 mM, pH 8.4, 0.8 ml $min^{-1}$) was passed with a wet nitrogen streaming (120 ml $min^{-1}$) into the reservoir.

**2.5 Removal of Endogenous Ammonia** Since principle of the proposed system is based on measurement of ammonia produced in the enzyme-catalyzed reaction, so endogenous ammonia molecules in the sample result in interfering with the determination of correct concentration of urea. In fact, it is known that rice wine includes about 6 ~ 10-fold concentration of ammonia in comparison to that of urea. So, ion exchange resins were applied to remove endogenous ammonia. Four milliliter sample solution of urea (0.3 mM) including ammonium chloride (3.0 mM), which diluted with 50 mM citrate buffer pH 5.0, was added to the ion exchange resins (3.0 g) and shaken softly for 3 minutes. The supernatant of the solution shaken with the resins was injected into the sensing system followed by filtration.

**2.6 Influence of Ethanol on the Measurement of Urea Using the FIA System** Before the evaluation of effect of ethanol on measurement of urea using the FIA system, we

investigated the tolerance of acid urease column against an ethanol. One hundred microliter of 50 mM citrate buffer solutions containing 0.5 mM urea and 5 % ethanol was injected into this FIA system more than 100 times, and then, the acid urease activity was measured.

After the evaluation of tolerance of the acid urease column against an ethanol, we evaluated influence of ethanol on determination of urea using the FIA system. For the evaluation, 0.3 mM ammonium chloride solutions containing various concentrations of ethanol were injected into this system.

**2.7 Determination of Urea in the Rice Wines** Real samples for determination of urea by this FIA system were randomly selected from commercially available rice wines. The rice wine samples were prepared by treatment of ion-exchange resins followed by 10-fold dilution with 50 mM citrate buffer solutions. An F-kit method which was conventionally used for determination of urea was employed for a comparative study.

## 3. Results and Discussion

**3.1 Measurement of Ammonia** Before the determination of urea, each of ammonium chloride solutions with various concentrations were injected into the sensing system without the enzyme column. An absorbance at 596 nm ($\Delta A$) of Thymol Blue solution was changed due to ammonia diffusion across the PTFE tubing in the gas diffusion unit. As shown in Fig. 2, a good linear relationship between the concentrations of ammonium chloride solutions and the peak height (changes in absorbance) was obtained. Therefore, various concentrations of ammonium chloride solutions were further injected and measured by this system. The good linearity was obtained in a range of 7.81 – 250 μM and the coefficient was calculated to be 0.999 (data was not shown). The response to ammonium chloride was obtained within 3 min. A relative standard deviation of change in absorbance ($\Delta A$) at the each concentration of ammonium chloride was about 2.8 % ($n = 5$).

**3.2 Determination of Urea** In order to evaluate the detection range of the system for urea measurement, various concentrations of standard urea solutions were injected into this system. Catalytic hydrolysis of urea by acid urease under acidic conditions is as follows:

$$\text{Urea} + 2\,H_2O + H^+ \xrightarrow{\text{Acid Urease}} 2\,NH_4^+ + HCO_3^-$$

Ammonium ions formed were detected by the FIA system. Twenty millimolar urea solution was prepared in 50 mM citrate buffer (pH 5.0) and then diluted (from 1.0 mM to 7.8 μM) with the same buffer. Each of 100 μl of these urea standards was injected into the sensing system and ammonium ions released by the enzymatic hydrolysis were measured. The coefficient was calculated to be 0.999 and the relative standard deviation for urea

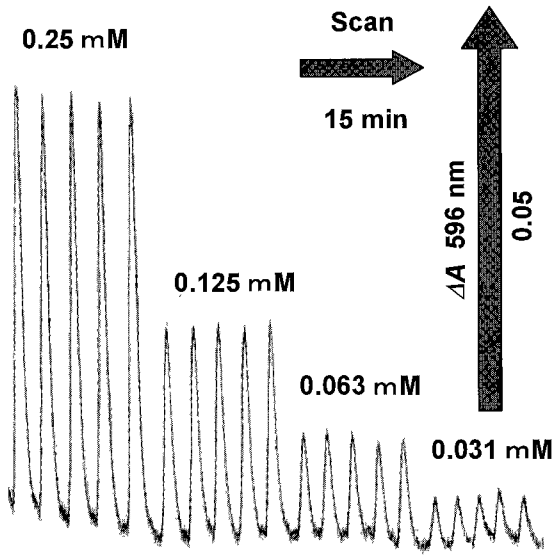


Fig. 2. Response curves to 100 μl injections of ammonium chloride standards with various concentrations.

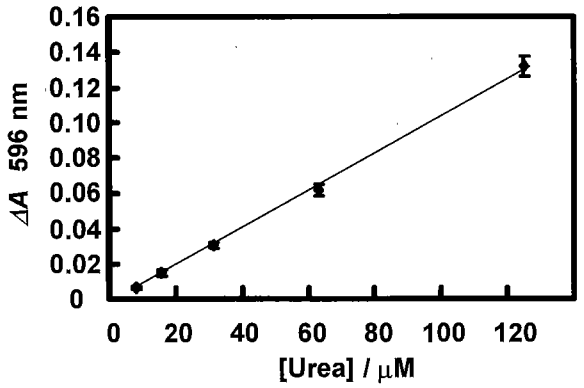


Fig. 3. Calibration graph for urea. The absorbance output was monitored using the FIA system. Concentrations of urea standards were prepared at 125, 62.5, 31.25, 15.63, 7.81 μM, respectively.

Table 1. Substrate specificity of the acid urease.

| Sample | Relative activity (%) | Contents in rice wine mM |
|---|---|---|
| Urea | 100 | n.d. |
| Ala | - | 3.5 |
| Arg | 4.4 | 2.2 |
| Asn | 1.8 | - |
| Asp | - | 2.2 |
| Gln | 1.6 | - |
| Glu | - | 2.9 |
| His | - | 0.5 |
| Ile | - | 1.6 |
| Leu | - | 2.4 |
| Lys | - | 1.2 |
| Phe | - | 1.4 |
| Pro | - | 3.5 |
| Ser | - | 1.9 |
| Thr | - | 1.1 |
| Tyr | - | 1.3 |
| Val | - | 2.7 |
| L-Ascorbate | - | 0.004 |

n.d. = no data, - = not detected.

determination with each concentration was about 3 % ($n$ = 5), and the lower limit of detection was 7.8 μM (Fig. 3). One assay took 7 min or shorter. Changing a range of detection gave a linear relationship in a range of 125 μM – 1.0 mM. Therefore, it was observed that a dynamic range for urea with this FIA system should be located between 7.8 μM and 1.0 mM. On the other hand, the lower limit of a quantitative analysis of urea by an F-kit for ammonia and urea as a conventional method is about 340 μM. Thus, this system enabled to determine significantly trace urea with good precision.

**3.3 Substrate Specificity of Acid Urease Column** The substrate specificity of acid urease from *L. fermentum* was investigated. Various amino acids which were much observed in rice wine were selected and the 100 μl of the 5.0 mM amino acid solution were injected into the immobilized acid urease FIA system. The relative activities for various amino acids were listed in Table 1. All amino acids were not hydrolyzed by acid urease except three amino acids which were arginine, asparagine, and glutamine. It is known that there are about 20 kinds of amino acids which were components of proteins. Asparagine and glutamine are known as an amido type amino acid, and there are no amino acids except these two amino acids. Table 1 shows that asparagine and glutamine were hydrolyzed to release ammonia by acid urease. Therefore, we considered that acid urease had weak amidase activity. Moreover, the table indicates that acid urease also acted on arginine which has a amidine structure. Although it is not clear how acid urease reacts on arginine, we consider that acid urease also has a weak arginase or arginine deaminase activity.

**3.4 Removal of Endogenous Ammonia** The influence of the ion exchange resins for removal of endogenous ammonia on the photometric responses was investigated following by measurement of their adsorptive activity for ammonium ions. Four milliliter of ammonium chloride solution (5.0 mM) was added to 3.0 g of the resins in a centrifugal glass tube and shaken softly for 3 minutes. Then the supernatant was injected to the assay system. Furthermore, 4.0 ml of the ammonium chloride solution was added to the same ion exchange resins, again. This manipulation was repeatedly performed until an ammonium chloride from the supernatant by this system should be detected. No noticeable peak was observed until injection of a supernatant of 6th 4.0 ml portions of the 5.0 mM ammonium chloride solution which was previously treated with the ion exchange resins. Therefore, 24 ml of 5.0 mM ammonium ions corresponding to about 120 μmoles of ammonium ions could be removed by this treatment (data was not shown). Then, the ion exchange resins were used to remove ammonium ions selectively from urea-containing samples. The selective removal of ammonium ions was tested against a sample containing of 0.3 mM urea and 3.0 mM ammonia. The sample was added to the ion exchange resin layer and followed by the same procedures. As seen in Fig. 4, there was no appreciable difference between 0.3 mM urea standard without the treatment (sample A) and the pre-treated sample (Sample B, C). Since sample B was same concentration of urea as sample A and sample C also included 0.3 mM urea and 3.0 mM ammonium chloride, 10-fold ammonium ions against urea was removed selectively by this method.

From these results, it was confirmed that this treatment method using the ion exchange resins might be useful to remove endogenous ammonia selectively from urea-containing solutions.

Moreover, this method was applied to the removal of arginine, asparagine and glutamine responding to acid urease. Five milimolar of those amino acids solutions were treated with ion-exchange resins and then injected into the FIA system. No responses were obtained by injection of those treated amino acids solutions (data was not shown).

**3.5 Influence of Ethanol on the Response of the FIA System** Tolerance of the immobilized acid urease *per se* to ethanol was evaluated before influence of ethanol on the response should be tested. One hundred microliter of ethanol-free urea solution was introduced into this system followed by more than 100 times injection of 100 μl of 5.0 % ethanol solutions. As shown in Fig. 5, no noticeable difference was exhibited between

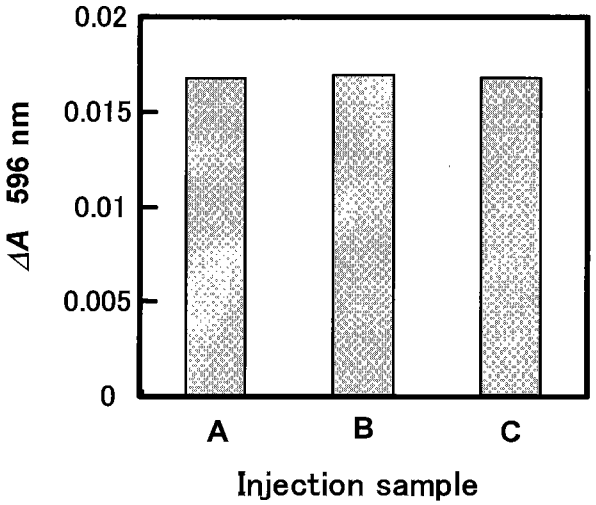


Fig. 4. Selective removal of ammonia from the urea-containing samples. The absorbance was measured by the FIA system. Sample A : 0.3 mM urea solution. Sample B : 0.3 mM urea solution followed by treatment of ion exchange resins. Sample C : 0.3 mM urea solution contained 3.0 mM ammonium chloride after treatment of ion exchange resins.

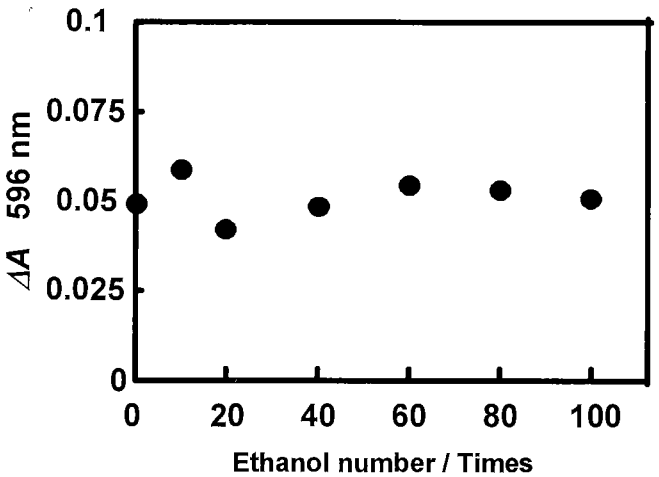


Fig. 5. Tolerance of acid urease column to ethanol. The absorbance was measured by the FIA system. A100 μl of 50 μM urea solution containing of 5.0 % ethanol were injected into the FIA system.

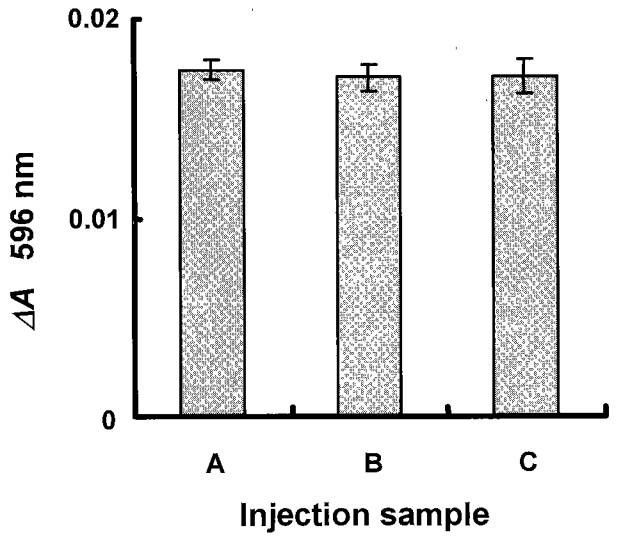


Fig. 6. Effect of ethanol against the proposed FIA system for urea determination. Absorbance was measured by this FIA system. Sample A : 0.3 mM ammonium chloride solution containing 1 % ethanol. Sample B : Same concentration of ammonium chloride solution as sample A containing 3 % ethanol. Sample C : Same as sample B except ethanol concentration, which concentration was 5 %.

Table 2. Comparison with the F-kit method and the proposed method for urea determination in rice wines.

| | Evaluated concentration of urea (mM) | |
|---|---|---|
| Sample | F-kit method | Proposed method |
| A | 0.30 | 0.23 |
| B | 0.33 | 0.34 |
| C | 0.20 | 0.20 |
| D | 0.29 | 0.22 |
| E | 0.46 | 0.43 |
| F | n.d. | n.d. |

the response of pre- and post-injection of 5.0 % ethanol solutions. Further investigation for ethanol tolerance of acid urease was tested by injection of 100 μl of 25 % ethanol solutions. Though the acid urease column were exposed to ethanol solutions more than 10 times, the acid urease did not show any changes in the activity before or after exposure of ethanol (data was not shown). This result means that the immobilized acid urease is remarkably tolerant of ethanol.

To investigate the influence of ethanol on the response, preparative urea solutions containing ethanol were injected into the FIA system. Standard urea solutions (0.3 mM) with several concentrations of ethanol (1, 3, 5, 10, 15 %) were injected. The value of the absorbance tended to increase as ethanol concentration was getting higher. We suspected that these results were due to the change of permeability of PTFE membrane by ethanol. The responses of all samples didn't correspond to the response in which 0.3 mM urea solution without ethanol was injected. Therefore, to reduce the effect of ethanol on the membrane, we added a small amount of ethanol to carrier prior to evaluate the effect on the FIA system. A 100 μl of 0.3 mM ammonium chloride solutions containing ethanol was injected into the system. As shown in Fig. 6, the responses of this system against sample B and C were good agreement with that of sample A. It means that this FIA system did not suffer from ethanol by adding ethanol to carrier previously.

**3.6 Determination of Urea in Rice Wine** From these results, we considered this FIA system might be promising for determination of urea in real samples. So, we applied the FIA system to determine a urea in 6 kinds of rice wines. After treatment with the ion exchange resins for removing endogenous ammonia, concentrations of urea in these real samples were measured using the FIA system. Trace urea in 5 samples was detectable and the contents of urea in these samples were below 0.5 mM. As indicated in Table 2, the concentrations of urea in real samples evaluated by this method were compared with those evaluated by the F-kit method. Correlation coefficient between concentrations evaluated by these methods was 0.95. According to the description in the manual, the lower limit of detection with the F-kit method was about 0.3 mM. On the other hand, it was 7.8 μM with the proposed method. Therefore, when the concentrations evaluated more than 0.3 mM were selected, the correlation coefficient between these concentrations was 0.99.

## 4. Conclusion

In this study, we propose a useful determination method for urea under acidic condition by using the immobilized acid urease column·FIA system. This FIA system enabled us to determine urea in the concentration ranging from 7.8 μM to 1.0 mM. This sensitivity is sufficient to determine urea in rice wine. Furthermore, considering the results that the acid urease and the FIA system was not influenced by ethanol, and that endogeneous ammonia could be successfully removed by treating with the ion-exchange resins, this FIA system should be promising to provide an effective determination of urea in alcoholic beverages. In fact, by determination of urea in real samples, this FIA method in combination with the immobilized acid urease should be a powerful tool for analysis of urea in rice wines.

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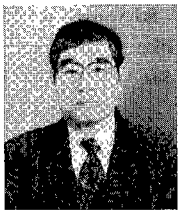
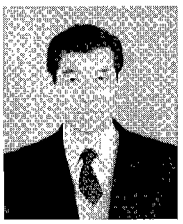
**Kunio Matsumoto** (Non-member) was born in Tokyo, Japan, on  March 27, 1942. He received a Ph. D. degree in Agriculture from The University of Tokyo in 1996. He has been employed by Toyo Jozo Co. Ltd. and subsequently Asahi Chemical Industry Ltd. He is presently a professor at Kanagawa Institute of Technology since 1996. He has worked on enzyme engineering, screening of microorganisms and enzymes. Japan Society for Bioscience, Biotechnology, and Agrochemistry, Japanese Society of Enzyme Engineering, The Chemical Society of Japan, The Japan Society for Analytical Chemistry, The Society for Bioscience and Bioengineering, Japan, The Society of Chemical Engineers, Japan, member.

**Ikuo Satoh**  (Member) was born in Tokyo, Japan, on September 29, 1947. He received a Ph. D. degree in Engineering from Tokyo Institute of Technology in 1977, and is presently a professor at Kanagawa Institute of Technology since 1995. He has worked on analysis of biomaterials by using immobilized enzymes and development of flow-injection biosensing. Japan Society for Bioscience, Biotechnology, and Agrochemistry, The Chemical Society of Japan, The Electrochemical Society of Japan, The Society for Analytical Chemistry, member.