

Flow Injection Analysis of Trace Urea in Alcoholic Beverages with an Acid Urease Column

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Abstract

A spectrophotometric flow-injection analysis (FIA) with an acid urease column was applied to determination of trace urea in alcoholic beverages.

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 a 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. Citrate buffer (50 mM, pH 5.0) as the carrier solution was continuously pumped through the system. Sample solutions were introduced into the system via a rotary injection valve. Ammonium ions formed in the enzymatic hydrolysis of urea were mixed with alkaline buffer (100 mM sodium phosphate, pH 12.0), and the mixed solution was sent to the gas diffusion unit consisting of a double tubing structure. Coloring reagent solution (Thymol Blue) streams in the Teflon tubing became dark-colored due to ammonia gas diffusion across the porous Teflon tubing, and subsequent increase in absorbance at 596 nm attributable to the reaction was successively monitored.

A wide, linear relationship was obtained between the concentration of urea and the variation in absorbance. The dynamic range of urea with use of this system was 7.8 μ M – 1.0 mM. Then, effect of an ethanol coexisting in the substrate solutions on the response was investigated. This system for the determination of urea did not noticeably suffer from any interferences due to the urea solution containing ethanol up to 5 % concentration.

These results demonstrate that this system should be promising to determination of trace urea in alcohol drinks.

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

1. Introduction

In Japan, sake is one of the most favorite alcoholic beverages, and it is drunk at cold and hot, or on the rocks and by cocktail, to say nothing of straight. Thus, sake is widely tasted in various styles. However, sake contains trace amounts of urea which was produced by yeasts during the fermentation of moromi mash [1]. Urea is considered as a precursor of ethylcarbamate [2], 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 [1]. Since ethylcarbamate has been known to be carcinogenic, teratogenic, and mutagenic [3, 4], removal of urea being a precursor of ethylcarbamate from sake is very important task to be solved urgently [2]. Recently, to solve this problem, application of acid urease to remove urea [1, 5] or use of a genetically engineered sake-yeast excreting no urea [6] has been investigated. Therefore, the monitoring system for evaluating urea removal simply and rapidly is required.

Because urea is one of useful chemicals as fertilizer [7, 8], feed [9, 10] and raw materials of synthetic resins [11, 12], analysis of urea has been widely investigated. In such analytical fields, many investigations of the determination of urea have been based on the measurement of the pH change or ammonia released from hydrolysis of urea by using urease catalysis. There are many reports with flow injection analyses based on electrochemical methods such as amperometry [13, 14], potentiometry [15, 16], conductometry [17, 18], calorimetry [19], and optical method such as absorptiometry [20], fluorescence

spectrophotometry [21-24], emission spectrophotometry [25-27]. All of these enzymatic methods for urea are using urease from jack bean, of which optimal pH is located in a range of 7.0 – 8.0. On the other hand, use of acid urease from *Lactobacillus fermentum* for determination of urea in sake is preferable against that of jack bean urease, because acid urease enables us to hydrolyze urea to ammonia and carbon dioxide even in lower pH region (optimal pH ranging from 4.0 – 5.0 [28]) and also is tolerant of alcohol. Therefore, we have developed a calorimetric determination system for urea with use of an acid urease as a recognition element [29, 30]. Furthermore, use of the acid urease for a urine analysis was not reported except these one. However, this calorimetric system was not always sufficiently sensitive to measure urea in sake. In this report, we proposed a novel determination system based on a photometric FIA (Flow-injection Analysis) system introducing gas diffusion device [31, 32] for improvement of the sensitivity of the urea determination by using an acid urease column as a recognition element.

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 μ m, inner diameter: 1 mm, outer diameter: 2 mm, length of part of gas diffusion layer: 120 mm) were purchased from F * I * A Instruments Co., Ltd. (Tokyo) and Flon Industry

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(Tokyo), respectively. Ion exchange resin (Amberlite MB-2, pore diameter; 300 - 850 μm) was obtained from Organo Corporation (Tokyo). Urea (biochemical grade) was from Wako Pure Chemical Industries, Ltd (Osaka, Japan). All other reagents were commercially available and of analytical grade.

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. Measurement of the soluble acid urease activity

The enzyme activity was measured based on an indophenol method. The enzyme preparation purified from NAGAPSHIN (50 mg) was dissolved with pure water at the concentration of 10 $\mu\text{g ml}^{-1}$. Five hundred microliter of this enzyme solution was added to 2.5 ml acetate buffer (0.1 M, pH 4.0) and a 1.0 ml urea solution (0.1 M) was also added to the mixture. The reaction mixture was allowed to stand at 37 $^{\circ}\text{C}$ for 30 min. Ten percent TCA (trichloroacetic acid, 4.0 ml) was added to the solution immediately after the incubation, and precipitates were filtered by Microfilter (AstroPore disc capsule type CACL 45 2.5 CMD, pore size: 0.45 μm ; Fuji Photo Film Co. LTD, Tokyo). Then, the filtrates were 10-fold diluted by doubly deionized water. Two milliliter of coloring reagent A (0.11 M phenol, 0.19 mM sodium nitroprusside in pure water) and of coloring reagent B (0.25 M sodium hydroxide, 1.5 % sodium hypochlorite solution diluted by pure water) were added to a 4.0 ml of the filtrates, and incubated for 30 min at 37 $^{\circ}\text{C}$. Absorbance at 640 nm of the reactant was measured with a spectrophotometer (UV-120-02, Shimadzu Corporation, Kyoto, Japan). Blank test of this enzyme activity was performed as follows. Equivalent amount of the enzyme solution (0.5 ml) and 2.5 ml acetate buffer were added to 4.0 ml of 10 % TCA. One milliliter of 0.1 M urea solution was added to the mixture followed by incubation of the enzyme reactant for 30 minutes. This solution was diluted to ten-fold with pure water and then, the 4.0 ml diluent was colored by coloring agent A and B (incubated for 30 minutes at 37 $^{\circ}\text{C}$) as a blank sample.

2.4. Immobilization of acid urease

Acid urease was covalently immobilized onto alkylaminated CPG as described previously [33]. The immobilized preparations were packed into a small polymer column and then mounted in a water-jacked holder.

2.5. 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 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.).

Citrate buffer (100 mM, pH 5.0) as the carrier solution (0.4 ml min^{-1}) was successively pumped through the system. Sample

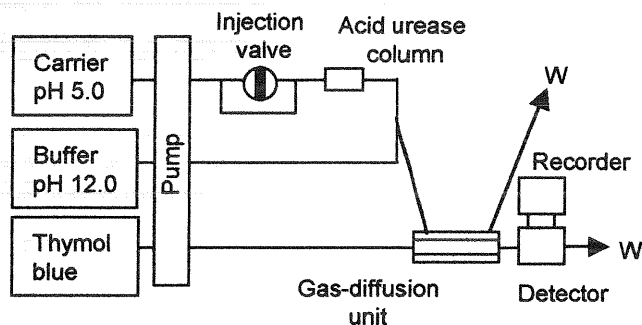


Fig. 1. System for urea determination

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. 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 due 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.

2.6. Removal of endogenous ammonia

Since principle of the proposed system is based on measurement of ammonia produced by the enzyme-catalyzed reaction, so endogenous ammonia molecules in the sample results in interfering with the determination of correct concentration of urea. In fact, it is known that sake 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 (0.5 mM) including ammonium chloride (4.0 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.

2.7. Influence of ethanol on the response of the FIA system

To evaluate influence of ethanol on the response of the FIA system, 0.1, 0.3, 0.5 mM urea solutions containing 2.5, 5.0, 10.0 and 15.0 vol. % of ethanol were prepared with 50 mM citrate buffer (pH 5.0), respectively. These solutions were injected into the presented system.

3. Results and Discussion

3.1. Determination of soluble acid urease activity

Hydrolysis of urea catalyzed by acid urease under acidic conditions is as follows:



To determine the enzyme activity, a calibration curve of concentration of standard ammonium sulfate versus the absorbance at 640 nm was prepared according to the method with ammonium sulfate instead of the enzyme solution (Fig. 2). The calculated coefficient was 1.000, and the regression equation was $y = 0.0128x$. The relative standard deviation for ammonium sulfate determination with each concentration was

below 0.5 %. The enzyme activity was determined to be 36.7 U mg⁻¹ protein.

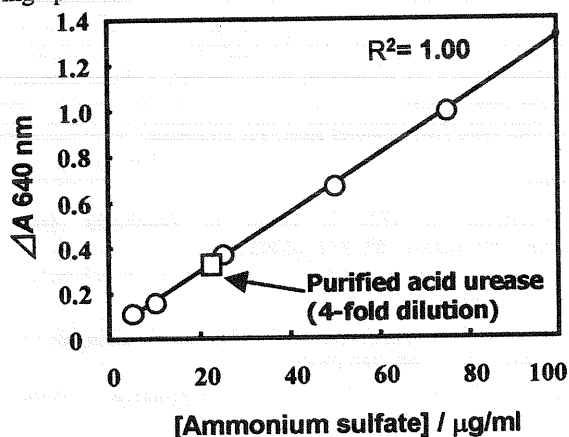


Fig. 2 Determination of soluble acid urease activity.

3.2. Determination of Ammonia

In order to evaluate the detection limits of the system before the determination of urea, each of ammonium chloride solutions with various concentrations were injected into the sensing system without the enzyme column. The change in absorbance at 596 nm (ΔA) of Thymol Blue solution colored by ammonia diffusion across the PTFE tubing in the gas diffusion unit was shown in Fig. 3. The baseline drift due to variation of concentration of HCO_3^- in Thymol Blue was observed, whereas, a linear relationship between the concentration of ammonium chloride and the absorbance was obtained. Therefore, ammonium chloride solutions with much lower concentrations were 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 (ΔA) at the each concentration of ammonium chloride was about 2.8 % ($n = 5$).

3.3. Determination of urea

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 catalysis were measured. The produced ammonium ions were converted to gaseous ammonia molecules

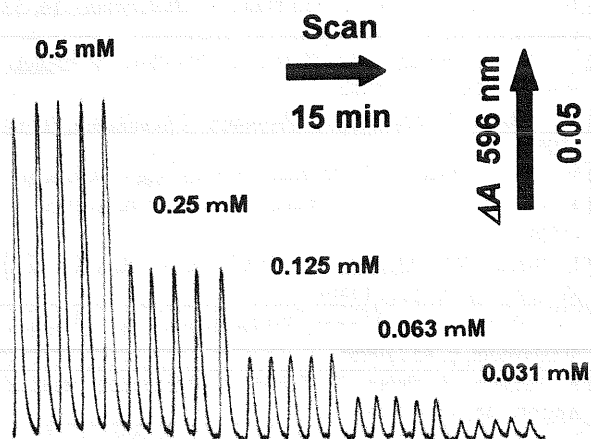


Fig. 3 Response curves to 100 μl injections of ammonium chloride standards with various concentrations.

and were monitored for determination of urea. While measurement of the produced HCO_3^- may be the other candidate for the index of urea determination, monitoring of ammonium ions have advantages that the produced amount is twofold against HCO_3^- and they are not susceptible to be effected on monitoring by contamination of the constituent of the air.

The coefficient was calculated to be 0.999 and the relative standard deviation for urea determination with each concentration was about 3 % ($n = 5$), and the lower limit of detection was 7.8 μM . Changing a range of detection gave a linear relationship in a range of 125 μM – 1.0 mM. Therefore, a determination range for urea with this FIA system was determined to be in a range of 7.8 μM to 1.0 mM. On the other hand, the lower limit of a quantitative analysis of urea by an F-kit (Nippon Beehringer Ingelheim Co., Ltd. (Tokyo)) for ammonia and urea as a conventional method is about 340 μM . Therefore this system enabled to determine significantly trace urea with good precision.

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 and the result was shown in Fig. 4. 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. 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 0.5 mM urea and 4.0 mM ammonia. The sample was added to the ion exchange resin layer and followed by the same procedures. There was no appreciable difference between 0.5 mM urea standard without the treatment and the pre-treated sample. Since the sample included 0.5 mM

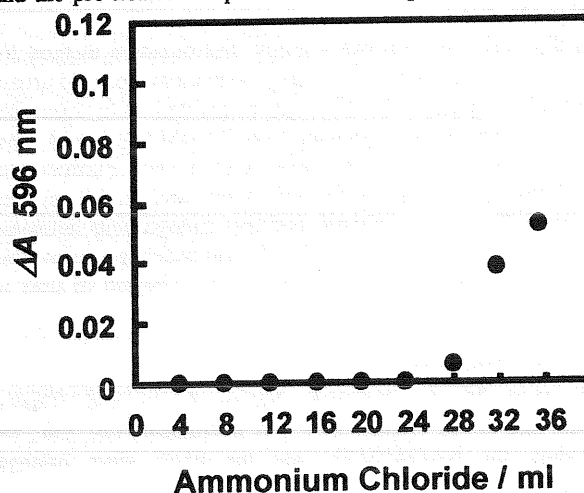


Fig.4 Removal of ammonium ions using the ion exchange resins (3.0 g). Ammonium chloride solution pretreated with ion exchange resin was injected into this FIA system.

urea and 4.0 mM ammonium chloride, 8-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 available to remove endogenous ammonia selectively from urea solutions.

3.5. Influence of ethanol on the response of the FIA system

To investigate the influence of ethanol on the response, preparative urea solutions containing ethanol were injected into the FIA system. As seen in Fig. 5, each of the response increased with increasing the ethanol concentration in the range of 5.0 – 15.0 %. However, no noticeable difference between responses of the samples with less than or equal to a 5 % concentration of ethanol and those of the ethanol-free samples was observed. Therefore, in the case of determination of urea in sake using this system, the real sample should be diluted to 3–5-fold with the carrier solution prior to injections. From the viewpoint on the sensitivity, the proposed system should be promising to determine urea in alcoholic beverages.

Tolerance of immobilized acid urease *per se* to ethanol was evaluated before influence of ethanol on the response was tested. One hundred microliter of ethanol-free urea solution was introduced into this system followed by more than 10 times injection of 100 μ l of 25 % ethanol solutions. No noticeable difference was exhibited between the responses of pre- and post-injection of 25 % ethanol solutions (data was not shown). This result means that the immobilized acid urease is remarkably tolerant of ethanol.

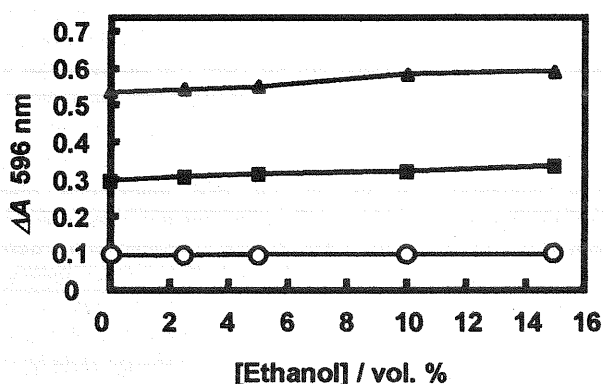


Fig.5 Effect of ethanol on the response of this FIA system. Urea concentration; ▲: 0.5 mM, ■: 0.3 mM, ○: 0.1 mM.

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 μ M to 1.0 mM. This sensitivity is sufficient to determine urea in sake. 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 the ion-exchange resins, this FIA system should promise effective determination of urea in alcoholic beverages.

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