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Fluorometric determination of urea in alcoholic beverages by using an acid urease column-FIA system

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Abstract

An acid urease column was applied to a fluorometric flow-injection analysis (FIA) system as a recognition element for determination of urea in rice wines.

The acid urease has specific properties of showing its catalytic activity in low pH range and tolerance to ethanol in comparison to those of a urease from jack-beans. The enzymes were covalently immobilized onto porous glass beads with controlled pore size and then, packed into a small polymer column. The flow-type of the biosensing system was assembled with a sample injection valve, the immobilized enzyme column, and a flow-through quartz cell attached to a fluorescent spectrophotometer. 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. A standard urea solution was measured through monitoring variations in fluorescent intensity attributable to fluorescent isoindole derivatives formed by coupling with ammonia molecules released in the enzymatic hydrolysis of urea and orthophthalaldehyde reagents. The fluorescent intensity was measured under the conditions of λ_{ex} = 415 nm and λ_{em} = 485 nm. A wide, linear relationship was obtained between the concentration of urea (1.0–100 μM) and the variation in fluorescent intensity. The monitoring did not suffer from ethanol and various amino acids contained in rice wines. Real samples pretreated with ion exchange resins for removal of endogenous ammonia were introduced into the FIA system and urea in the samples was determined. These results were compared with those obtained with use of an F-kit method. The proposed FIA system should present sensitive, selective and convenient analysis of urea in alcoholic beverages.

Keywords: FIA; Acid urease; Urea; Rice wine; Fluorometry

1. Introduction

Since early times, alcoholic beverages have been tasted around the world and many kinds of alcohol drinks such as wine, whisky, brandy and so on have been commercially available. In Japan, rice wine, in Japanese sake, is one of the most favorite alcoholic beverages. Alcohol drinks are manufactured in microbial fermentation process and many byproducts are contained. Urea is one of the byproducts which is undesirable because urea is considered as a precursor of ethylcarbamate [1]. The ethylcarbamate has been known to be carcinogenic, teratogenic, and mutagenic [2,3]. This reaction is promoted by heating under acidic condition, distillation and long-term storage [4]. Therefore, removal of urea in rice wine is required [1]. In fact, application of acid urease to remove urea [4,5] or use of a genetically engineered sake-yeast producing no urea [6] has been tested. Thus, a monitoring system for urea in rice wine is desired. Most of these investigations on the determination of urea have been based on the measurement of changes in ammonia enzymatically released from urea. There are a great number of reports on flow-injection analyses with electrochemical methods such as amperometry [7,8], potentiometry [9,10], conductometry [11,12] and optical methods such as absorptiometry [13–15], fluorescence spectrophotometry [16–19], emission spectrophotometry [20–22] and calorimetry [23,24]. In all of these reports for determina-

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tion of urea, urease from jack-beans has been used. However, in the case of determination of urea in alcohol beverages like rice wine, acid urease from *Lactobacillus fermentum* is preferable against the urease from jack-beans. The acid urease can exhibit its catalytic activity even in lower pH region, and also have an alcohol tolerance and a significant stability. In previous study, we reported a determination system based on photometry for urea in rice wine by using an acid urease column as a recognition element [25,26]. However, much more sensitive and selective determination system was required. Therefore, in this study, we would like to demonstrate the sensitive and selective determination method for urea in rice wine by using a fluorometric system with 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). The enzyme was purified from the NAGAPSHIN by desalting after ultrafiltration. The acid urease solution was lyophilized (FREEZE DRYER FDU-830, EYELA) to obtain acid urease powder. The purified preparations were covalently immobilized onto CPG. Details of the methods were described previously [26]. The immobilized preparations were loaded in a small polymer column (Tosky Corporation, Tokyo: 10 mm length, 7.0 mm diameter, packed volume, 300 μl). Controlled-pore glass (CPG, mean pore diameter 24.2 nm, particle size 120–200 mesh) was purchased from Funakoshi Co., Ltd. (Tokyo). An F-kit (TC Ammonia) was purchased from Nippon Boehringer 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) and *o*-phthalaldehyde (OPA) were from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and Sigma-Aldrich Japan K.K. (Tokyo), respectively. 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. Flow system and procedure

A schematic diagram of the flow system is shown in Fig. 1(A). The system was assembled with two double-plunger pumps (Sanuki DM3M-2044 for carrier and alkaline reagent, DMX-2000 for OPA reagent, Sanuki Industry Co., Ltd., Tokyo), a rotary injection valve with a 100 μl sample loop (Sample Injection Valve, Cat. No. 5020; Rheodyne Inc., California, USA), the immobilized acid urease column (300 μl) surrounded by the water jacket maintained at

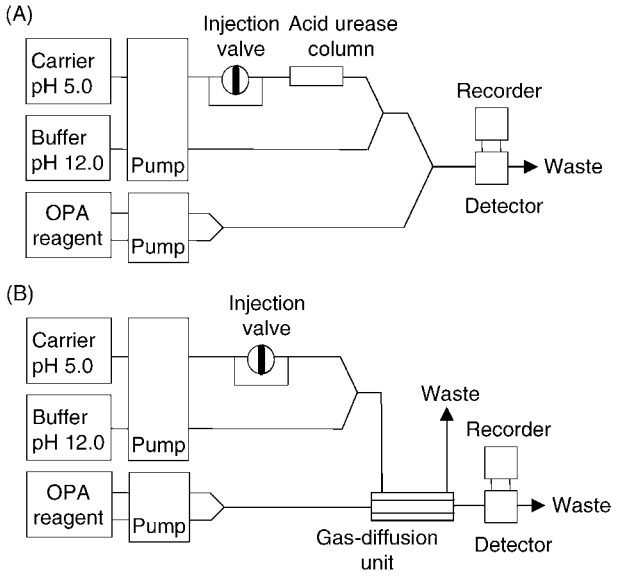
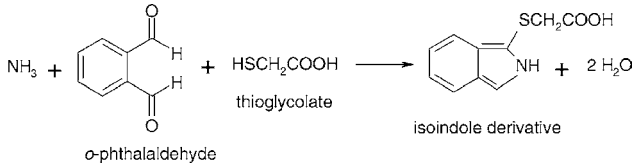


Fig. 1. Schematic diagram of a flow-injection system for the determination of urea using an immobilized acid urease column. (A) FIA system used in this study, (B) FIA system with gas-diffusion unit used in previous report.

303 K, a flow-type of fluorometer (821-FP, JASCO Corporation, Tokyo), and a pen recorder (Multi-Pen Recorder; type R-62M3, Rikadenki Kogyo Co. Ltd., Tokyo).

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. The carrier line which contained ammonium ions formed in the enzymatic hydrolysis of urea was joined with the line of strongly alkaline buffer (gas-diffusion buffer: 100 mM sodium phosphate, pH 12.0), and the mixed solution (0.8 ml min^{-1}) was connected with a flow stream of OPA reagent (OPA: 20 mM, thioglycorate: 30 mM, sodium tetraborate: 4 mM, pH 11.6). The OPA reagent solution was passed with a wet nitrogen gas stream (120 ml min^{-1}) to keep the reagent pH value stable.

A 100 μl of standard urea solutions with various concentrations was separately prepared and injected into the flow system. OPA reagent was reacted with ammonia molecules released in the enzymatic hydrolysis of urea, and a fluorescent isoindole derivative was produced in the coupling reaction. The fluorescent intensity of isoindol derivatives (λ_{ex} = 415 nm and λ_{em} = 485 nm) was successively monitored through the flow-type of fluorometric spectrophotometer. A relationship between concentrations of standard urea solutions and fluorescent intensities was investigated and thus, carribration was carried out. The reaction was shown in following formula.



2.3. Determination of urea in rice wine with a conventional method

An F-kit was used for the validation. Urea in a rice wine was hydrolyzed to form ammonia and carbon dioxide in the presence of urease contained in the kit. The liberated ammonia reacted with 2-oxoglutarate to produce L-glutamate in the presence of glutamate dehydrogenase, which was also contained in the kit and also reduced type of nicotinamideadenin dinucleotide (NADH). The amount of NADH oxidized in the above reaction was stoichiometrically equal to the amount of ammonia. NADH is determined by means of its absorbance changing at 340 nm. From above principle, concentrations of urea in rice wines were determined. Practical method of the F-kit was referred to the manual.

3. Results and discussion

3.1. Determination of urea

In order to evaluate detection limits 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:

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

The 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 100 to 1.0 μM) with the same buffer. Each of 100 μl of these urea standards was injected into the sensing system and ammonium ions released in the enzymatic hydrolysis were measured. As shown in Fig. 2, a good linear relationship between the concentrations of urea solutions and the peak height (variation in fluorescent intensity) was obtained. Therefore, various concentrations of urea solutions were further injected and measured with this system. The relative standard deviation for urea determination with each concentration was about 3% (n = 5), and one assay of a sample injection took 7 min or shorter. The dynamic range for urea with this FIA system was between 1.0 and 100 μM. On the other hand, the lower limit of a quantitative analysis of urea with 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.

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

Tolerance of the immobilized acid urease per se to ethanol was reported previously [26].

In order to investigate an effect of ethanol on the response, 100 μl of 30 μM ammonium chloride solution containing several concentrations of ethanol (1, 3, 5, 10, and 15%) were injected into an FIA system illustrated in Fig. 1(B) (the column-free). And the effect of ethanol on the responses of the FIA system was compared with the effect of ethanol on those of FIA system using gas-diffusion unit (GDU) described in previous report [26]. As shown in Fig. 3, the fluorescent intensity was increased as the ethanol contents were increased by using the FIA system with GDU. We suspected that these results should be due to the change in permeability of the PTFE membrane against ethanol. On the other hand, no effect of ethanol on the fluorescent intensity could be observed by using the proposed FIA system, which was free from the GDU. Moreover, when a solution at same concentration of ammonium chloride was injected, the fluorescent intensity obtained with use of the proposed FIA system was considerably higher than that in the FIA system with GDF (previously used system).

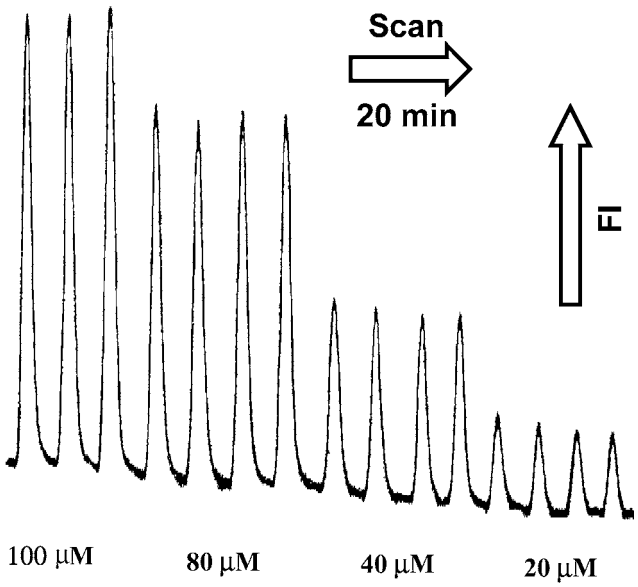


Fig. 2. Response curves to 100 μl injections of urea standard solutions with various concentrations.

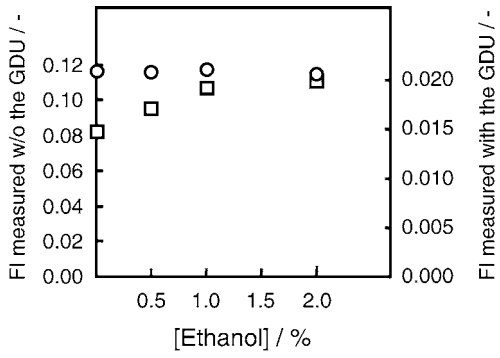


Fig. 3. Effect of ethanol on the OPA reagent. ◯: various concentrations of ethanol solutions containing 30 μM NH_4Cl were injected into the FIA system (without a gas diffusion unit) □ . Various concentrations of ethanol solutions containing 30 μM NH_4Cl were injected into an FIA system with a gas diffusion unit.

3.3. Selectivity of OPA reagent and substrate specificity of the acid urease

It is well known that there are many kinds of amino acids in rice wines. If the OPA reagent would react with those amino acids, the FIA system without the gas diffusion unit could not be used for determination of urea in real samples. Therefore, we tested the selectivity of the OPA reagent by introducing various amino acids into the proposed FIA system without acid urease column. In addition, if the acid urease reacts with the amino acids and thereby releases ammonia, it may be difficult to determine urea in real samples. So, we tested the selectivity of OPA reagent and the substrate specificity of the acid urease by injecting various types of amino acids into the FIA system.

As seen in Table 1, the selectivity of the OPA reagent against various kinds of amino acids was very high and only three kinds of amino acid, threonine, leucine, and glutamine, exhibited a slight response to the OPA reagent.

In contrast, the relative activities of the acid urease for various amino acids were listed in Table 1 and compared with that of urease from jack bean. Several amino acids were hydrolyzed by acid urease, which were arginine, asparagine, aspartic acid, glutamine, and histidine. These amino acids will hardly effect the determination except for argentine, which has an amidine structure. It is indicated that the acid urease also has a weak arginase or arginine deaminase activity. On the other hand, in the case of urease from jack bean, most of amino acids in Table 1 were not or slightly catalyzed by the urease. We consider that acid urease was more suitable for a determination of urea in such amino acids contained in samples.

Table 1
Selectivity of OPA reagent and substrate specificity of the acid urease and urease

Sample	Relative activity (%)			Contents in rice wine (mM)
	OPA reagent	Acid urease	Urease	
Urea	–	100	100	0.2–0.5
NH_4Cl	100	–	–	1–5
Ala	–	–	3.1	3.5
Arg	–	10.6	1.9	2.2
Asn	–	1.4	2.0	n.d.
Asp	–	1.0	1.8	2.2
Gln	0.27	1.1	1.5	n.d.
Glu	–	–	2.2	2.9
Gly	–	–	5.1	3.9
His	–	1.2	5.4	0.5
Ile	–	–	1.8	1.6
Leu	0.14	–	2.5	2.4
Lys	–	–	n.d.	1.2
Phe	–	–	4.8	1.4
Ser	–	–	n.d.	1.9
Thr	0.25	–	n.d.	1.1
Tyr	–	–	n.d.	1.3
Val	–	–	4.7	2.7
Pro	–	–	n.d.	3.5
Met	–	n.d.	n.d.	0.27
Trp	–	n.d.	n.d.	0.049

–, not detected; n.d., no data.

3.4. Removal of endogenous ammonia

This FIA system for urea is based on measurement of ammonia formed in the enzyme-catalyzed reaction. Therefore, 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. Two-milliliter sample solution of urea (0.3 mM) including ammonium chloride (3.0 mM) was diluted with 2 ml citrate buffer (50 mM, pH 5.0) and then, added to the ion exchange resins (3.0 g) and shaken softly for 3 min. The supernatant of the solution shaken with the resins was injected into the sensing system followed by five-fold dilution with citrate buffer. As seen in Table 2, there was no noticeable difference between 30 μM urea standard (sample A) and the pre-treated samples (Sample B, C), of which difference was below 3%. Since the urea concentration in sample B was same as those in sample A and sample C also including 30 μM urea and 300 μM ammonium chloride at the final concentration, about 10-fold ammonium ions against urea was thus removed selectively with use of this method.

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

Moreover, this method was applied to the removal of arginine and the other responsive amino acids to acid urease. One mM sample of those amino acids solutions were treated with the ion-exchange resins and then injected into the FIA system. No responses were observed by injecting those amino acids solutions preliminary treated (data was not shown).

3.5. Determination of urea in rice wine

From these results, we considered this FIA system might be applicable to determination of urea in real samples. So, we applied the FIA system to determine urea in 10 kinds of real rice wines by using standard addition method. After treatment with the ion exchange resins for removing endogenous ammonia, concentrations of urea in these real samples were

Table 2
Removal of ammonium ions using the ion exchange resigns (3.0 g)

Sample	F1
A	0.175 ± 0.017
B	0.171 ± 0.018
C	0.176 ± 0.038

A, 30 μM urea solution was injected into the FIA system. B, 30 μM urea solution was injected into the FIA system after treatment of ion exchange resins. C, 30 μM urea solution containing 300 μM ammonium chloride solution pretreated with ion exchange resins was injected into this FIA system.

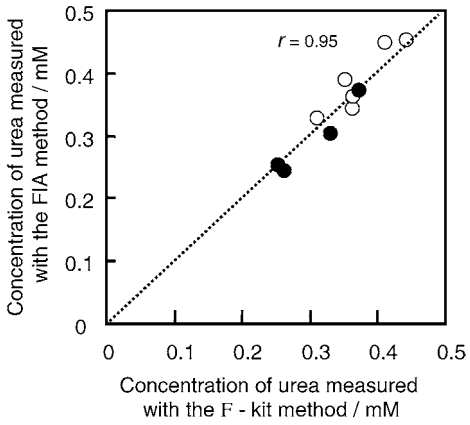


Fig. 4. A correlationship between concentrations of urea in rice wine by using of FIA method and that of F-kit method; ◯: concentration of urea in Junmai-shu, ●: concentration of urea in rice wine with alcohol additives.

measured using the FIA system. Among these 10 samples, six were Junmai-shu which was made of only rice, water and koji mold, and the other samples were not Junmai-shu which was made of rice, water, koji mold and a small amount of pure distilled alcohol. The average of concentration of urea in 6 kinds of Junmai-shu was 0.387 mM (Fig. 4, symbol: ◯). On the other hand, the average of concentration of urea in 4 kinds of rice wines with alcohol additives was 0.29 mM (Fig. 4, symbol: ●). These results indicate that Junmai-shu tends to contain a lot of urea in comparison with amount of urea in rice wine with alcohol additive.

Concentrations of urea in real samples evaluated by this method were compared with those evaluated by the F-kit method and the correlation between proposed method and standard F-kit method was shown in Fig. 4. Correlation coefficient between concentrations evaluated by these methods was 0.95. The recovery ratio calculated with the standard addition method was between 92.7 and 107.3% and the average was 97.6%. These results show that a good correlation between concentrations of urea in rice wine by using the FIA method and that of F-kit method was obtained.

4. Conclusion

In this study, we proposed a simple and sensitive determination method for urea using the immobilized acid urease column FIA system. This FIA system enabled us to determine urea in the concentration ranging from 1.0 to 100 μM. This sensitivity is sufficient to determine urea in real rice wine followed by ion exchange pre-treatment. Furthermore, considering the results 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, it was demonstrated that this FIA method in combination with the immobilized acid urease column should be a powerful tool for analysis of urea in commercially available rice wines.

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