Journal of Industrial Microbiology & Biotechnology (2001) 26, 363–368

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An improved method for determination of ethyl carbamate in Korean traditional rice wine

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An improved extraction method for ethyl carbamate, a genotoxic and carcinogenic compound found in various fermented foods and beverages, was investigated for its determination in the two most typical Korean traditional rice wines, *takju* and *yakju*. When the rice wines were extracted twice with chloroform at 30°C for 60 min, the recovery of ethyl carbamate was less than 16%. When they were saturated with NaCl before extraction, the recovery of ethyl carbamate increased to 24.4% in *takju* and 67.2% in *yakju*. Adjustment of pH to 9.0 after NaCl saturation in *takju* resulted in a dramatic increase of recovery to 81.2%, but not in *yakju*. When the contents of ethyl carbamate and its precursor, urea, in various Korean traditional rice wines were determined, there was no correlation between the two contents. This is due to the fact that storage time is more important than urea content in the formation of ethyl carbamate in rice wine. In addition, its storage at high temperature resulted in a dramatic increase in ethyl carbamate content according to the prolonged storage time, suggesting that storage time and temperature play a key role in the formation of ethyl carbamate in Korean traditional rice wine. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 363–368.

Keywords: ethyl carbamate determination; rice wine; NaCl saturation; pH; storage; urea

Introduction

Ethyl carbamate ($NH_2COOCH_2CH_3$), also known as urethane, is a naturally occurring compound in all fermented foods and beverages where microbiological activity has been an integral part of the production process. It is a genotoxic compound *in vitro* and *in vivo*, and is a carcinogen in laboratory rats and mice [1,17,22,33]. Since the Liquor Control Board of Ontario, Canada detected trace levels of ethyl carbamate in various alcoholic beverages including several types of wine, whisky, and brandy in 1985, there have been a number of studies on the determination method and the level of ethyl carbamate in a variety of fermented foods and alcoholic beverages [3,5,12,24,33]. Ethyl carbamate was used as a therapeutic agent for humans during the 1940s, but these days, its usage has become prohibited due to its toxicity. Canadian health authorities prohibited the use of urea, a well-known precursor for ethyl carbamate in fermented foods and alcoholic beverages. In addition, a guideline of the level of ethyl carbamate in various alcoholic beverages has been established in Canada [33].

There have been two proposed mechanisms for ethyl carbamate formation in alcoholic beverages. One mechanism is a spontaneous chemical reaction between urea and ethanol in fermented and undistilled alcoholic beverages [18,20]. The other is the conversion of cyanides produced by thermal decomposition of cyanohydrins of isobutyl aldehyde to ethyl carbamate during or after distillation in distilled alcoholic beverages [16,23]. Urea, a precursor of ethyl carbamate, originates from arginine degradation by the reaction of arginase, a *CAR1* gene product, in *Saccharomyces cerevisiae* [27,28]. Alcohol yeasts could produce urea by degradation of arginine by arginase action during fermentation. The urea is then degraded to CO_2 and NH_3 by a multifunctional enzyme, urea carboxylase, and by allophanate hydrolase, which is a *DUR1,2* gene product [9,26]. The expression of the *DUR1,2* gene has been reported to be slightly induced by urea [30], whereas *CAR1* gene expression is induced at a high level in the presence of arginine [4,10,15,22]. Therefore, not all the urea formed by argininase action during alcohol fermentation by *S. cerevisiae* may be degraded and the residual urea could react with ethanol to convert to ethyl carbamate during and after heat treatment of alcoholic beverages. Therefore, recent research on ethyl carbamate has been focused largely on urea reduction in alcoholic beverages using acid urease [8,21] and the development of nonurea-producing alcohol yeast strains [14,25,29,31].

Nowadays, there is a general agreement that ethyl carbamate levels in fermented foods should be maintained at the lowest levels that are technically possible. In order to support this, it is essential to establish an optimum quantitative method for determining ethyl carbamate suitable for each alcoholic beverage. There are many quantitative methods for determining ethyl carbamate in fermented foods and alcoholic beverages [2–4,7,12,13,24,33]. However, the recovery of ethyl carbamate varies based on the types of fermented foods and alcoholic beverages. It also depends on various factors that occur during the extraction procedure. Specifically, the recovery of ethyl carbamate from a certain type of rice wine is less than 20% [7,13].

We have studied efficient extraction methods for ethyl carbamate from Korean traditional rice wines to improve the recovery of ethyl carbamate. The effects of storage time and

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Received 10 January 2001; accepted 17 March 2001

temperature on ethyl carbamate formation in rice wine were also investigated.

Materials and methods

Extraction of ethyl carbamate

Korean traditional rice wines were purchased in Taegu, Korea and were stored at 4°C. Extraction of the wines for determination of ethyl carbamate was carried out by the method of Ough [19]. Twenty milliliters of wine was transferred into a separatory funnel and extracted using 30 ml of chloroform. The mixture was then incubated without shaking for 3 min to allow the layers to separate. The chloroform layer was carefully collected and the sample was re-extracted with 30 ml of chloroform. The combined extract was dehydrated by passing it through anhydrous sodium sulfate and it was then concentrated to 5 ml on a rotary evaporator. The concentrated sample was loaded on a Florisil column containing 10 g activated Florisil capped with a layer of 3 g of anhydrous sodium sulfate. Ethyl carbamate was eluted from the column with 200 ml of a mixture of benzene/diethyl ether/methanol (108:32:1, v/v). Thirty milliliters of ethyl acetate was added in the eluate and its volume was reduced to about 2 ml on an evaporator. Finally, the sample was concentrated to 100 μl in a centrifugal concentration system and used as the sample for analysis of ethyl carbamate using a gas chromatograph-mass spectrometer (GC-MS).

Gas chromatography-mass spectrometric analysis of ethyl carbamate content

Ethyl carbamate was detected by selected ion monitoring at m/z 62 on a mass spectrometer (Micromass Ltd., Quattro II, Manchester, UK) coupled with a gas chromatograph (Micromass, Fisons GC 8000). A DBwax-bonded fused silica capillary column (0.25 mm×30 m, film thickness 0.25 μm) that interfaced directly with the mass spectrometer was used with the splitless injection mode. The other operation conditions for determination of ethyl carbamate content are shown in Table 1. Calibration of ethyl carbamate was carried out in the range of 0–100 μg l^{-1}. For determination of ethyl carbamate in various rice wines, propyl carbamate was used as an internal standard at a final concentration of 50 or 100 μg l^{-1}. Ethyl carbamate and propyl carbamate peaks appeared at around 12.0 and 13.3 min of retention time, respectively. It was confirmed that the area of each peak was proportional to the amount of ethyl carbamate and propyl carbamate injected. Typical GC-MS chromatograms of *takju* (to which 50 μg l^{-1} of propyl carbamate was added) and *yakju* (100 μg l^{-1} of propyl carbamate), the two typical Korean rice wines, for the determination of ethyl carbamate contents, are shown in Figure 1. In both cases, the ethyl carbamate peak appeared at around 12.0 min, whereas the peak of propyl carbamate, used as an internal standard, was eluted at about 13.3 min.

Ethyl carbamate recovery

Twenty milliliters of rice wine was mixed with 1 μl of ethyl carbamate (final concentration 50 μg l^{-1}), and the ethyl carbamate content was then analyzed as above after the wine was saturated with 8 g NaCl followed by pH adjustment to 9.0 in *takju* but no pH adjustment in *yakju* before extraction with chloroform. Corresponding rice wines without the addition of ethyl carbamate were also analyzed to determine the natural levels of ethyl carbamate. Recovery of ethyl carbamate was calculated as the percentage of the ethyl carbamate recovered by extraction with chloroform from the amount added to the wine. All data in tables and figures represent the average of at least two trials that were each performed in triplicate.

Determination of urea content

For determination of urea content in rice wine using urease, ammonium ion was first removed using a Dowex 50WX8-100 column as previously described [6]. Two hundred milliliters of wine was concentrated on an evaporator and distilled water was added to make the volume 10 ml. The concentrated solution was applied to a Dowex 50WX8-100 column and urea was eluted with distilled water to remove ammonium ion bound to the resin. The eluate was then concentrated and distilled water was added to make the total volume 10 ml, which was used for determination of the urea content at 530 nm using urease by the method of Gutmann and Bergmeyer [11]. The mixture of 0.2 ml of the final concentrated solution, 0.1 ml of urease solution (2 mg ml^{-1} in 50% glycerol), and 0.4 ml of 1/15 M sodium phosphate buffer (pH 7.5) in a test tube was incubated at 37°C for 15 min. After incubation, 5 ml of 0.106 M phenol–0.17 mM sodium nitroprusside and 5 ml of sodium hypochlorite were added to the tube, which was then incubated at 37°C for 30 min to visualize the ammonia formed from urea by the urease action. Its optical density

Table 1 GC-MS parameters for determination of ethyl carbamate contents

Item	Operation condition
Gas chromatography	
Instrument	Fisons GC 8000 series
Carrier gas	He, 1.5 ml/min
Column	DBWax (30 m×0.25 mm), film thickness: 0.25 μm
Temperature gradient	70°C held for 2 min, 8°C/min to 150°C, 20°C/min to 250°C, 250°C for 10 min
Injector	210°C
Detector	210°C
Mass spectrometry	
Instrument	Micromass Quattro II
Fragmentation mode	Electron Impact at 70 eV
SIM	m/z 62
Injection mode	Splitless

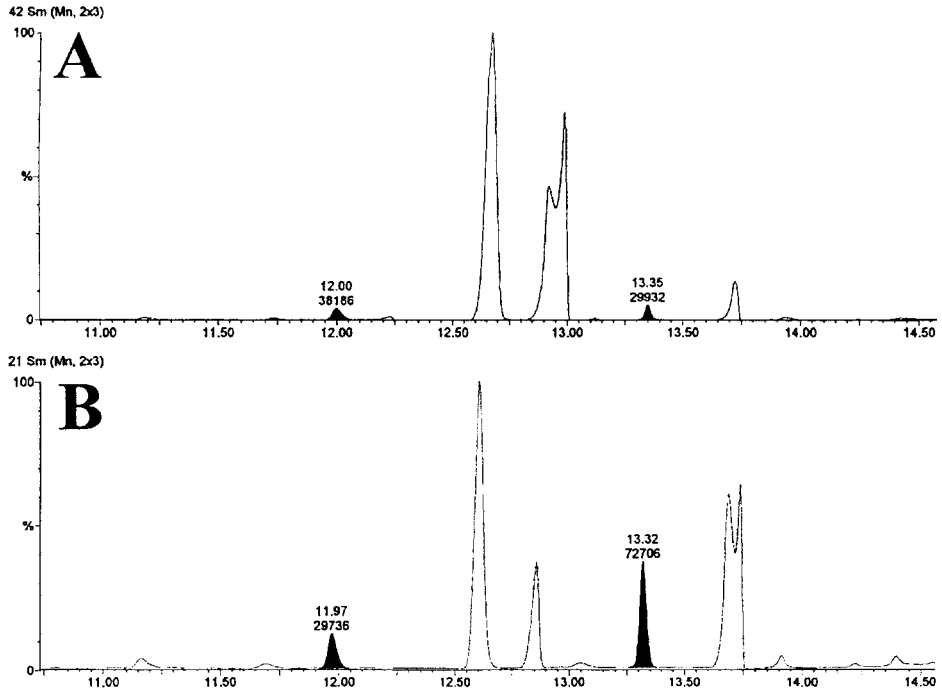

Figure 1 Typical GC-MS chromatograms of rice wines. The two most typical Korean traditional wines, *takju* (A) and *yakju* (B), were tested for their ethyl carbamate content via a GC-MS (Micromass). Propyl carbamate was added as an internal control at final concentrations of 50 $\mu g\,l^{-1}$ in *takju* and 100 $\mu g\,l^{-1}$ in *yakju*.

was measured at 530 nm and urea content was calculated as mg l^{-1} from a standard curve.

Results and discussion

Effect of extraction solvents, temperature, and shaking time on the recovery of ethyl carbamate

Most published methods for the determination of ethyl carbamate in alcoholic beverages use either chloroform or dichloromethane for extraction, which are the two most efficient extraction solvents for ethyl carbamate [24]. Therefore, we first tried those two organic solvents for extraction of ethyl carbamate from Korean traditional rice wines and then compared the recovery of ethyl carbamate after its content in the extracts was determined on a GC-MS. When dichloromethane was used for the extraction according to the method of Cairns *et al.* [5], the recovery of ethyl carbamate was only 4.7%. The extraction with chloroform under the conditions of the original method described by Ough and Trioli [21] resulted in 9% recovery (data not shown). Similar results have also been reported in the case of Korean rice wine with other extraction solvents [7,13] where recovery of ethyl carbamate was less than 20% when ethyl acetate was used as the extraction solvent. Therefore, we investigated factors affecting the recovery of ethyl carbamate from Korean rice wine using chloroform as an extraction solvent. To determine the effect of temperature and shaking time for the extraction of ethyl carbamate by chloroform, the extraction was carried out at 20, 30 and 40°C for 1 h as well as at 37°C for 30 min, 1 and 2 h. In addition, the other procedures followed exactly the original method shown in Materials and Methods section. As shown in Table 2, the recovery of ethyl carbamate was the highest at 30°C, which was about 15.6%, but dropped to 7.0% at 40°C. When the extraction was carried out for 1 h, the highest recovery of ethyl carbamate was achieved and further extraction resulted in no increase in recovery.

Effect of NaCl saturation on the recovery of ethyl carbamate

Because of the poor recovery of ethyl carbamate by the extraction method above, we tried to optimize the extraction conditions to improve the recovery ratios. First, we tested the effect of salt saturation on the recovery of ethyl carbamate (Figure 2). Eight grams of NaCl was added and dissolved in 20 ml of rice wine for 30 min by shaking. Once saturated, ethyl carbamate was extracted from the sample using chloroform. When the sample was saturated

Table 2 Effect of extraction conditions on the recovery of ethyl carbamate

Condition		Recovery (%)
Temperature (°C)[a]	20	12.4
	30	15.6
	40	7.0
Time (min)[b]	30	11.5
	60	15.6
	120	14.3

Ethyl carbamate was extracted twice with chloroform at 20, 30, and 40°C for 1 h[a] or at 30°C for 30, 60 and 120 min[b] from 20 ml of wine, *yakju*, with or without the addition of 1 μl of ethyl carbamate (50 $\mu g\,l^{-1}$). Recovery of ethyl carbamate was calculated as the percentage of the ethyl carbamate recovered by the extraction from the amount added in the rice wine. All data in this and the other tables and figures represent the average of at least two trials that were each performed in triplicate. Maximum variations were ±10%.

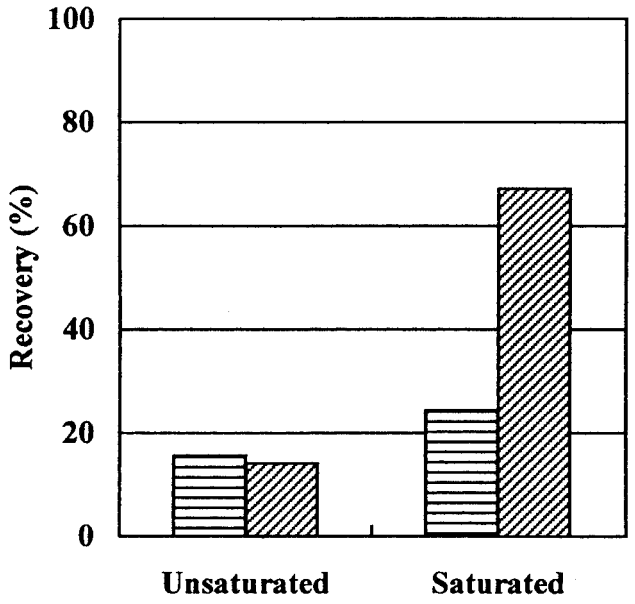

Figure 2 Effect of NaCl saturation of rice wine on the recovery of ethyl carbamate. Twenty milliliters of two typical Korean traditional rice wines, *takju* (▤) and *yakju* (▨), was saturated with 8 g of NaCl. Then ethyl carbamate was extracted with chloroform.

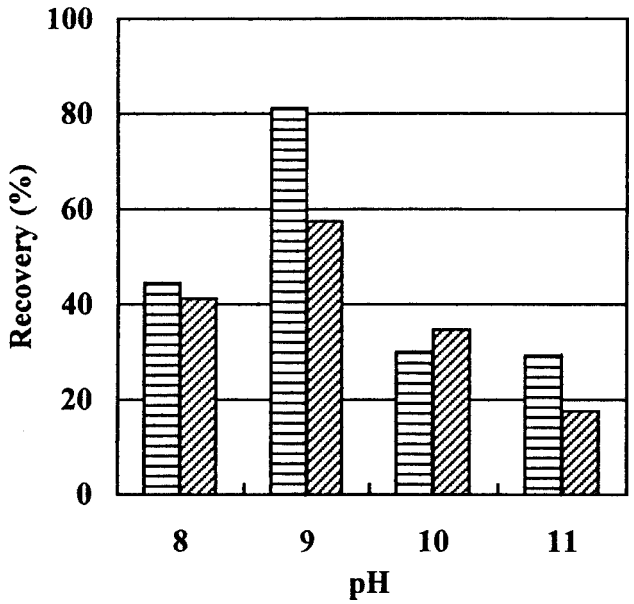

Figure 3 Effect of pH adjustment of rice wine on the recovery of ethyl carbamate. The pH of *takju* (▤) and *yakju* (▨) was adjusted with 0.5 N NaOH to the range shown in the figure after saturation with NaCl. Then ethyl carbamate was extracted from the rice wine with chloroform. The original pH in both rice wines was 3.9.

with NaCl, the recovery increased dramatically in *yakju* to 67.2% compared to 15.5% in the case of the unsaturated sample (Figure 2). However, a slight increase in recovery was obtained in *takju*, from 15.5% when unsaturated to 24.4% when saturated.

Ethyl carbamate is highly soluble in water, about 2 g ml^{-1} at 25°C [33]. Since its solubility in organic solvents is generally less than that in water, salt saturation of the sample might result in an increase in the recovery of ethyl carbamate with chloroform extraction. According to Chung and Kwon [7] and Kim *et al.* [13], the recovery of ethyl carbamate was much higher in Korean soy sauce that contains about 18% NaCl than in rice wine. It has also been reported that addition of NaCl in distilled spirits could increase the recovery of ethyl carbamate to 75% [2]. This is thought to be due to the fact that ethyl carbamate becomes more insoluble and can be extracted into the chloroform layer more efficiently when the rice wine is saturated with salts. Thus, NaCl saturation has great potential for the efficient extraction of ethyl carbamate in other wines.

Effect of pH on the recovery of ethyl carbamate

NaCl saturation resulted in a dramatic increase in the recovery of ethyl carbamate in *yakju* but not in *takju*. Therefore, the effect of pH adjustment in *takju* on the recovery of ethyl carbamate was investigated (Figure 3). The original pH in both rice wines was about 3.9. They were adjusted to the pH range of 8.0–11.0 with 0.5 N NaOH after NaCl saturation. Each sample was then extracted twice with chloroform and then analyzed for its ethyl carbamate recovery. Further procedures exactly followed those described in Materials and Methods section. Figure 3 shows the effect of pH adjustment on recovery of ethyl carbamate. On the recovery significantly increased by the pH adjustment in *takju*, and the maximum recovery (81%) was obtained when the pH was adjusted to 9.0. This might be due to the fact that ethyl carbamate loses its positive charge in the amino group in the alkaline pH range and thus becomes more insoluble in water. Adjustment of pH in grape wines to the alkaline range before extraction with dichloromethane resulted in an increase in the recovery of ethyl carbamate as well [12]. However, no increase in the recovery of ethyl carbamate was obtained in the *yakju*. Preliminary experiments showed that the pH adjustment in the acidic range has no effect on the recovery of ethyl carbamate (data not shown). As a result, it was thought that NaCl saturation followed by pH adjustment to 9.0 in *takju* or just NaCl saturation in *yakju* is important to improve recovery of ethyl carbamate. Accordingly, two different types of the Korean traditional wine showed big differences in the pattern of ethyl carbamate recovery under the extraction conditions such as NaCl saturation and pH adjustment. *Takju* and *yakju*, the two most typical Korean rice wines, are made from steamed starch materials (mainly rice) by the simultaneous enzymatic saccharification and fermentation by *S. cerevisiae* [32]. However, there are two differences in the brewing of the two rice wines. One is the use of saccharifying agents for each rice wine. The Japanese type of koji is used for *takju*, which is prepared by growing *Aspergillus kawachi* in steamed rice. For brewing of *yakju*, *nuruk*, the unique Korean koji, is used which is made of ground wheat by growing various natural

Table 3 Contents of urea and ethyl carbamate in Korean traditional rice wines

Wine	Sample	Urea (mg l^{-1})	Ethyl carbamate (μg l^{-1})
Takju	BR	2.84	46.13
	KS	2.46	53.35
Yakju	KH	5.23	35.86
	SS	6.84	38.78

Two typical samples each for *takju* and *yakju* were obtained in a commercial market, Taegu, Korea and were tested for their urea and ethyl carbamate contents.

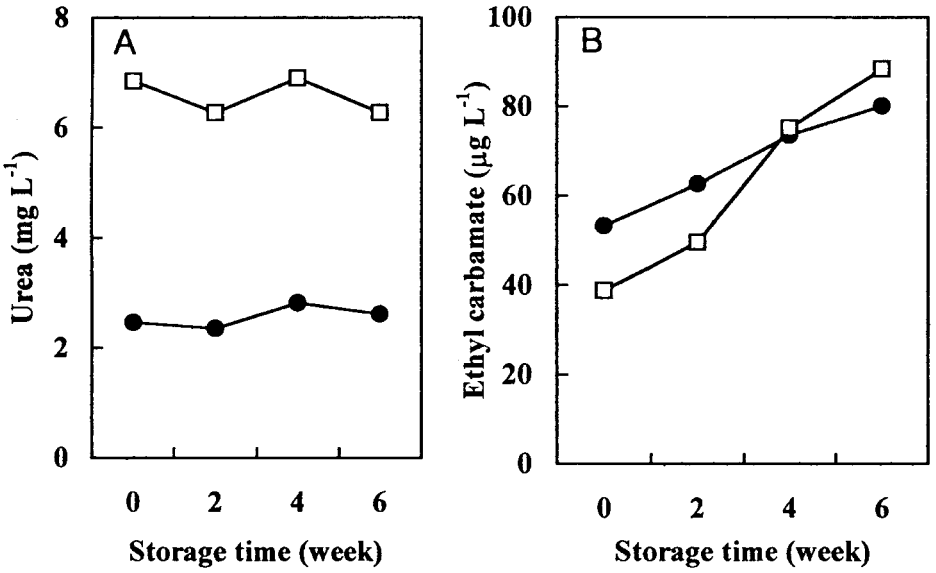

Figure 4 Changes in the urea and ethyl carbamate contents in rice wine during storage. *Takju* (●) and *yakju* (□) were stored at 30°C for 6 weeks and their urea (A) and ethyl carbamate (B) contents were monitored every 2 weeks during storage.

molds that produce amylases. The other difference is the filtration process for each wine. *Yakju* is generally prepared by filtration through diatomaceous earth after the solids are removed from the fermented mixture by a filter press to produce a clarified wine. *Takju* is prepared by removal only of big particles from the fermented mixture through a rough screen, whose pore size is around 30 mesh, to produce a turbid wine containing small particles. However, it cannot yet be explained whether these differences affect the patter of ethyl carbamate recovery under the conditions used in this study.

Occurrence of ethyl carbamate in Korean traditional rice wine

Four kinds of Korean rice wine (two samples each for *takju* and *yakju*) were tested for the determination of their ethyl carbamate contents using the extraction method developed in this study and their urea content as well (Table 3). The urea contents in *takju* (2–3 mg l^{-1}) were lower than those in *yakju* (5–7 mg l^{-1}). This is thought to be due to *takju* containing 6%, v/v, of ethanol, was more diluted from the fermented mixture than was *yakju* containing 13%, v/v, of ethanol. However, ethyl carbamate contents were 26–53 μg l^{-1} in *takju* and 35–39 μg l^{-1} in *yakju*, indicating *takju* containing a lower amount of urea has a higher level of ethyl carbamate than *yakju*. Ethyl carbamate is formed from the reaction of urea, a precursor for ethyl carbamate, and ethanol in alcoholic beverages [3,16,18,20].

These results prompted us to analyze the effect of storage time on the formation of ethyl carbamate in rice wines. *Takju* and *yakju* were stored at 30°C for 6 weeks and changes in their urea and ethyl carbamate contents were monitored every 2 weeks (Figure 4). No significant changes in the urea contents were found in either *takju* or *yakju* during the storage (Figure 4A). However, ethyl carbamate contents increased significantly with prolonged storage time. The increase was more rapid in the *yakju* containing a higher level of urea than *takju* containing a lower level of urea (Figure 4B, compare their urea contents in Table 3). These results suggest that more ethyl carbamate formation occurred in rice wines containing higher levels of urea during storage, but its storage time is a more important factor in ethyl carbamate formation than its urea content. In addition, the ambiguous result shown in Table 3, that the *takju* containing a lower amount of urea had a higher level of ethyl carbamate than the *yakju* was thought to be due to the difference in their storage time.

In order to test the effect of storage temperature on ethyl carbamate formation, a similar experiment to that shown in Figure 4 was carried out using a fresh rice wine. Wine was taken from the brewing company and stored at various temperatures for 6 weeks

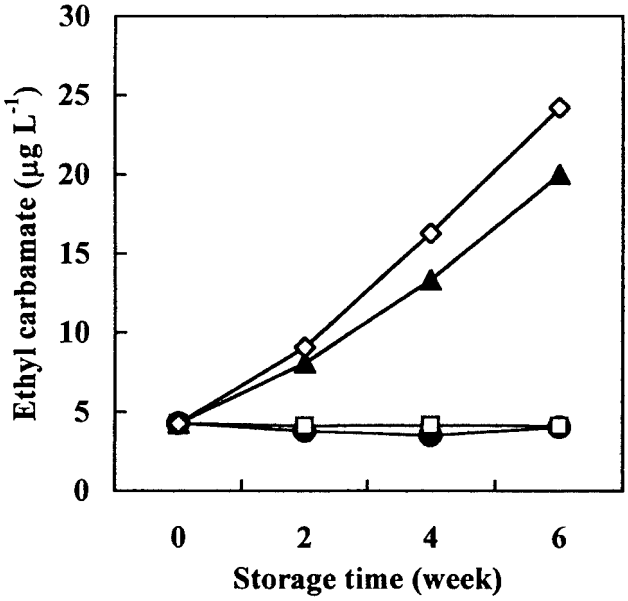

Figure 5 Effect of storage temperature on ethyl carbamate formation in rice wine. Fresh wine containing 4.3 μg l^{-1} of initial ethyl carbamate content was stored at various temperatures, 4 (●), 10 (□), 20 (▲), and 30°C (◇) for 6 weeks and its ethyl carbamate content was monitored every 2 weeks during storage.

and the changes in ethyl carbamate content were monitored every 2 weeks during the storage (Figure 5). The initial level of ethyl carbamate content was 4.3 $\mu g\, l^{-1}$, which is very low compared to those in the rice wine shown in Table 3. No increase in the ethyl carbamate content was found during storage below 10°C. However, during storage at 20°C or 30°C, the ethyl carbamate content increased dramatically. The increase at 30°C was more rapid than at 20°C. The highest level of ethyl carbamate (24.2 mg l^{-1}) was obtained after storage for 6 weeks at 30°C. Accordingly, the results obtained in this study argue that fresh rice wine contains a very low level of ethyl carbamate. In addition, its storage time and temperature as well as its urea content participate in the formation of ethyl carbamate.

Acknowledgement

This work was supported by a research grant (No. 297023-3) from the Ministry of Agriculture and Forestry, Korea.

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