

Ethyl Carbamate Formation from Cyanate in Model System of Ethanol-Water Media Using Response Surface Methodology

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ABSTRACT: Ethyl carbamate (EC) has been identified as a possible human carcinogen belonging to Group 2A. EC is naturally formed during the fermentation and storage of alcoholic drinks and fermented foods. When ingested in large amounts, EC can cause various health problems, such as gastroenteric hemorrhage, vomiting, and cancer. In this study, optimization of EC formation from cyanate was examined using response surface methodology (RSM), a central composite design that includes variables such as alcohol concentration (10, 15, 20, 25, and 30%), pH (2.5, 3.0, 3.5, 4.0, and 4.5), storage temperature (5, 10, 15, 20, and 25°C), and storage duration (2, 4, 6, 8, and 10 days). EC content was determined using gas chromatography with flame ionization detection and the results were optimized using RSM. EC formation from cyanate degradation was found to increase with storage duration and temperature, acidity, and alcohol concentration. Cyanate degradation was associated with the formation of EC. Approximately 83.1±0.1% of cyanate was degraded to 538±9 µM of EC. However, not all of the cyanate reacted with ethanol during fermentation to form EC. This study aimed to develop the ideal conditions for EC analysis to reduce EC production in alcoholic drinks and fermented foods.

Keywords: alcoholic drinks, cyanate, ethyl carbamate, model system, response surface methodology

INTRODUCTION

All fermented foods such as bread, soy sauce, yogurt, olives, and alcoholic drinks including beer, wine, whiskey, brandy contain ethyl carbamate (EC) (Battaglia et al., 1990; Beland et al., 2005; Weber and Sharypov, 2008; Ryu et al., 2015; Bai et al., 2017). In the 1940s, EC was used as a hypnotic in humans and an anesthetic in animals. However, in 1943 it was reported to be genotoxic and carcinogenic in mice (Baffa et al., 2011; Zhao et al., 2013). Thereafter, it was found to be lethal to various animal species, particularly mice, rats, hamsters, and monkeys (Beland et al., 2005). The International Agency for Research on Cancer categorized EC into Group 2B, i.e., probably carcinogenic to humans (Lachenmeier et al., 2012; Pflaum et al., 2016; Mohapatra, 2018). Moreover, EC is rapidly absorbed from the gastrointestinal tract and skin of the human body into the blood (European Food Safety Authority, 2007). Previous studies have reported alcoholic beverages as among those foods that contribute to high EC intake in humans (Ryu et al., 2015).

The assessment of EC content and cyanide degradation

in fermented foods and alcoholic drinks has been performed worldwide using various methodologies and technologies (Galinaro et al., 2015). The primary precursors of EC production have been recognized as a carbamyl group containing urea, citrulline, and carbamoyl phosphate. In addition, cyanic acid and diethyl pyrocarbonate play a role in EC synthesis (Zhou et al., 2019). In spirits, particularly those made using stone fruit, cyanate is the predominant precursor of EC (Riachi et al., 2014). Most cyanogenic glycosides in stone fruit seeds can be enzymatically or thermally degraded into hydrocyanic acid that is then converted to cyanate, which in turn reacts with ethanol to form EC (Pflaum et al., 2016). Several mechanisms have been developed to reduce EC levels in alcoholic drinks to determine its genesis and improve control practices that minimize EC formation during storage (Leça, 2014); these include the use of processed products, antioxidants, and genetically modified yeasts (Gowd et al., 2018). EC levels can be reduced by either preventing or disintegrating EC precursors.

Currently, there is no worldwide consensus on the maximum quantity of EC in fermented foods and alco-

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holic drinks; however, some countries have set their own standards. For instance, in 1985, Canada imposed EC limits for the first time after high EC levels were found in alcoholic drinks; the maximum amounts set for wine and fruit brandy were 30 and 400 µg/L, respectively. The United States of America (USA) has introduced voluntary restrictions for domestic alcoholic drinks (Nowak et al., 2013). Countries such as the Czech Republic, Brazil, France, Germany, and Switzerland follow the Canadian limits, and South Korea has only set the maximum EC limit for table wine as 30 µg/L (Ryu et al., 2015). To date, government regulations are yet to be introduced in South Korea to regulate allowable EC levels in alcoholic drinks other than table wine. Given that EC levels have yet to be extensively studied, restricted tests have been performed to track EC levels in alcoholic beverages using robust rapid test processes. Therefore, it is important to determine a consistent EC standard for alcoholic drinks to provide appropriate guidelines for current and future EC regulations.

Recently, response surface methodology (RSM) has been used to assess the safety of production in the food industry. RSM combines mathematical and statistical tools to determine the impact of many variables and optimizes various biotechnological procedures. It is also used as a numerical technique for relating product treatments to results and for establishing regression equations to define the interrelationships between input parameters and product features. Primarily, RSM involves using a series of carefully designed experiments to obtain an optimal response while reducing the number of time-consuming studies as well as lowering costs (Elibol and Ozer, 2002; Arslan-Alaton et al., 2009; Yi et al., 2010; Nair et al., 2014). The goal of the present study was threefold: (1) to develop a stable, selective, and responsive cyanate (OCN^-) analysis technique for use with ethanol-water mixtures; (2) to detect and quantify EC formation from cyanate using an ethanol-water media model system; and (3) to analyze the association between the formation of EC and the degradation of cyanate.

MATERIALS AND METHODS

Chemicals and materials

EC, butyl carbamate (BC) as an internal standard, sodium cyanate, and 2-aminobenzoic acid were supplied by Sigma-Aldrich Co. (St. Louis, MO, USA). Chloroform was obtained from Samchun Pure Chemical (Pyeongtaek, Korea), and ethanol was obtained from Honeywell Burdick & Jackson (Ulsan, Korea). Sodium hydroxide and citric acid were purchased from Junsei Chemicals (Tokyo, Japan). Hydrochloric acid was supplied by Daejung Chemicals and Metals (Siheung, Korea). All chemicals used in

this study were of analytical grade. Water was deionized using an ultra-pure and pure all-in-one water purification system (Dongwon Scientific Co., Seoul, Korea).

Experimental design

The experiment was developed using the Design-Expert statistical software (version 7.0.1; Stat-Ease Inc., Minneapolis, MN, USA). The RSM-central composite design was used in the experimental design process, which included four factors: storage temperature ($^{\circ}\text{C}$), storage duration (d), alcohol concentration (%), and pH (Table 1). These variables were tested at five distinct levels (-2 , -1 , 0 , $+1$, and $+2$) to investigate the interactions among the variables at different levels for two responses: EC generation and cyanate degradation (Table 2) (Arslan-Alaton et al., 2009).

Preparation of model solutions

The model solutions were prepared by mixing different ethanol concentrations (10, 15, 20, 25, and 30% v/v) and citric acid buffer at pH 2.5, 3.0, 3.5, 4.0, and 4.5 (Chen et al., 2017). The mixtures were spiked with sodium cyanate (NaOCN) and then mixed for 10 s using a vortex mixer (GW-92VM, Hwashin Tech Co., Ltd., Seoul, Korea). The solutions were then divided into several aliquots and transferred into appropriate conical tubes for further treatment. EC formation was allowed to occur based on the different values of two fermentation parameters: duration (2, 4, 6, 8, and 10 days) and temperature (5, 10, 15, 20, and 25°C). Working solutions were prepared at 1, 5, 10, 50, 100, 250, 500, and 1,000 µM by dilution with chloroform to prepare a standard curve (Kim et al., 2013).

Cyanate analysis

Cyanate was detected by mixing 1.0 mL aqueous ethanolic solution aliquots containing NaOCN with 1.0 mL of the 2-aminobenzoic acid mixture. Glass vials were used to store the obtained solutions; these were sealed using rubber septa and coated with tinfoil prior to being subjected to heat treatment at 40°C in an oven for 10 min. Subsequently, 2 mL of HCl (4 mol/L) was added, and the resultant solution was heated in an oven at 75°C for 10 min. At room temperature, the final solution containing quinazolinedione was cooled. After around 10 min, a

Table 1. Cooled levels of independent factors in the response surface methodology

| Factor | Level | | | | |
|--|-------|----|-----|----|-----|
| | -2 | -1 | 0 | +1 | +2 |
| Alcohol concentration (%) | 10 | 15 | 20 | 25 | 30 |
| Storage temperature ($^{\circ}\text{C}$) | 5 | 10 | 15 | 20 | 25 |
| Storage duration (d) | 2 | 4 | 6 | 8 | 10 |
| pH | 2.5 | 3 | 3.5 | 4 | 4.5 |

Table 2. Experimental values for the four variables of the central composite design and their responses in terms of cyanate degradation and ethyl carbamate (EC) formation

| Run | Factor 1 | Factor 2 | Factor 3 | Factor 4 | Response 1 | Response 2 |
|-----|----------|----------|----------|----------|----------------|----------------------|
| | | | | | Cyanate (%) | EC (μM) |
| 1 | 20 | 3.5 | 5 | 6 | 66.1 \pm 0.1 | 389 \pm 8 |
| 2 | 20 | 205 | 15 | 6 | 77.7 \pm 0.2 | 503 \pm 9 |
| 3 | 25 | 4 | 10 | 4 | 63.9 \pm 0.3 | 414 \pm 4 |
| 4 | 25 | 3 | 10 | 8 | 71.9 \pm 0.4 | 465 \pm 3 |
| 5 | 25 | 4 | 20 | 4 | 72.2 \pm 0.1 | 467 \pm 9 |
| 6 | 10 | 3.5 | 15 | 6 | 62.7 \pm 0.1 | 406 \pm 5 |
| 7 | 25 | 3 | 10 | 4 | 66.9 \pm 0.3 | 433 \pm 9 |
| 8 | 30 | 3.5 | 15 | 6 | 81.3 \pm 0.3 | 526 \pm 3 |
| 9 | 20 | 3.5 | 15 | 2 | 64.3 \pm 0.2 | 379 \pm 9 |
| 10 | 15 | 4 | 20 | 4 | 69.2 \pm 0.2 | 448 \pm 3 |
| 11 | 15 | 4 | 10 | 4 | 53.8 \pm 0.1 | 348 \pm 3 |
| 12 | 25 | 3 | 20 | 4 | 75.9 \pm 0.2 | 49 \pm 8 |
| 13 | 20 | 4.5 | 15 | 6 | 66.9 \pm 0.1 | 433 \pm 5 |
| 14 | 25 | 4 | 10 | 8 | 67.9 \pm 0.1 | 441 \pm 7 |
| 15 | 15 | 3 | 20 | 4 | 69.2 \pm 0.2 | 448 \pm 3 |
| 16 | 15 | 3 | 10 | 8 | 66.3 \pm 0.1 | 429 \pm 3 |
| 17 | 15 | 3 | 20 | 8 | 74.2 \pm 0.4 | 479 \pm 3 |
| 18 | 20 | 3.5 | 25 | 6 | 79.4 \pm 0.2 | 514 \pm 4 |
| 19 | 15 | 4 | 10 | 8 | 60.1 \pm 0.1 | 389 \pm 9 |
| 20 | 15 | 3 | 10 | 4 | 59.8 \pm 0.2 | 387 \pm 9 |
| 21 | 20 | 3.5 | 15 | 6 | 70.0 \pm 0.1 | 453 \pm 3 |
| 22 | 15 | 4 | 20 | 8 | 71.4 \pm 0.3 | 462 \pm 9 |
| 23 | 20 | 3.5 | 15 | 10 | 74.0 \pm 0.1 | 479 \pm 8 |
| 24 | 20 | 3.5 | 15 | 6 | 70.8 \pm 0.3 | 458 \pm 4 |
| 25 | 25 | 3 | 20 | 8 | 83.1 \pm 0.1 | 538 \pm 9 |
| 26 | 25 | 4 | 20 | 8 | 77.1 \pm 0.2 | 499 \pm 6 |

Values are presented as mean \pm SD of three replicates.

Factor 1, alcohol concentration (%); Factor 2, pH; Factor 3, storage temperature ($^{\circ}\text{C}$); Factor 4, storage duration (d).

fluorescence spectrophotometer (UV-1800; Shimadzu, Kyoto, Japan) was used at 310 nm to analyze the solution (Ohe et al., 2014). The cyanate concentration versus the emission values was plotted to create standard curves.

EC extraction

EC in all samples collected during the fermentation process was detected via direct injection into a gas chromatography system with a flame ionization detector (ACME 6100 GC-FID, YL Instrument, Anyang, Korea). Upon EC formation, the same volume of chloroform was added to each tube. Each mixture was strongly shaken using a vortex mixer (GW-92VM, Hwashin Tech Co., Ltd.) and centrifuged at 4°C and 10,000 rpm for 10 min using a Supra R22 centrifuge (Hanil Scientific Inc., Gimpo, Korea). Thereafter, aliquots were taken using a 10 mL syringe needle, and the samples were stored in glass vials (Kim et al., 2013). The EC from each sample in each glass vial was determined according to incubation duration and temperature; a 1 μL sample was taken from the glass vials using a 10 μL syringe, and the sample was injected into the gas chromatography system. The detection and quantification of EC formation due to cyanate degrada-

tion was determined by assessing the peak area obtained after injection.

EC analysis

Samples were directly injected into a gas chromatography system to determine EC concentrations. An SP-2560 fused silica capillary column (100 m \times 0.25 mm; 0.2 μm film thickness; Supelco Inc., Bellefonte, PA, USA) was used to separate EC. Temperatures at the injector and detector interfaces were maintained at 210°C and 260°C , respectively. The temperature of the gas chromatography oven was as follows: the initial column temperature was 150°C for 1 min, followed by a 2 min increase to 180°C at $15^{\circ}\text{C}/\text{min}$, and then a 4 min increase to 250°C at $10^{\circ}\text{C}/\text{min}$. At a flow rate of 1.5 mL/min, nitrogen gas was used as the carrier gas. In split mode, 2.0 μL aliquots of each sample were injected into the GC apparatus (Filley et al., 2002). BC was utilized as an internal reference to determine the EC level in the produced samples. The peaks for EC and BC were at 11.2 and 12.2 min retention times, respectively. The area of each peak was confirmed to be proportionate to the amount of EC and BC injected. EC was calculated as ppm of the EC production by extract-

ing the amount applied to the sample with chloroform. All data in the tables and figures are the average of at least two experiments, each of which was completed in triplicate.

Statistical and multivariate analyses

For data analysis of EC production and cyanate degradation, a central composite design of the RSM was applied via Design-Expert. Tables and model graphs with a 3-dimensional view were used to illustrate the data.

RESULTS AND DISCUSSION

Cyanate degradation

Analysis results for the models of cyanate degradation are summarized in Table 3. The values of cyanate degradation content calculated using the response surface quadratic model were positively correlated ($R^2=0.9102$). The adequate precision value or signal-to-noise ratio index in the cyanate model was 10.367, indicating that the model can be used to explore the design. A model must have a precision value of at least 4 to be considered a good fit for the data (Karimifard and Alavi Moghaddam, 2018). The F-value of the model was 7.97, suggesting that the cyanate degradation values obtained in this study under vari-

ous alcohol conditions, storage temperatures, duration, and pH were significant ($P<0.05$). In particular, the storage conditions had a substantial impact on cyanate degradation. “Prob>F” values of <0.05 indicate that the measured values are significant, whereas values of >0.10 are not significant. In this case, the measured values (designated as A for alcohol, B for pH, C for temperature, and D for time) were significant. With a strong positive coefficient ($R^2=0.91$) and low coefficient of variation ($CV=4.63$), the “lack of fit F-value” of 36.00 suggested that the lack of fit was not significantly related to the pure error. There is a 12.90% probability of a large lack of fit F-value occurring due to noise. However, a nonsignificant lack of fit value indicates that the model fits the data well.

Equation (1) can be used to predict cyanate degradation for given levels of each variable; it is useful to determine the relative effect of these variables, which is achieved by comparing the variable coefficients of the estimates shown in Table 3.

$$\begin{aligned} \text{Cyanate} = & 70.40 + 3.84A - 2.22B + 5.01C + 1.69D - 0.11AB \\ & - 0.39AC + 0.069AD + 0.42BC - 0.39BD - 0.16CD \\ & - 0.12A^2 - 0.045B^2 - 0.68C^2 + 0.42D^2 \quad (1) \end{aligned}$$

The values of the four independent variables, alcohol concentration, pH, storage temperature, and storage dura-

Table 3. Analysis of variance for the response surface quadratic model of cyanate degradation and ethyl carbamate (EC) formation

| Source ¹⁾ | df | Cyanate degradation ²⁾ | | EC formation ³⁾ | |
|----------------------|----|-----------------------------------|----------------------|----------------------------|----------------------|
| | | Estimated value | P-value | Estimated value | P-value |
| | | | Prov>F | | Prov>F |
| Model | 14 | 70.4 | 0.0007*** | 455.5 | <0.0001*** |
| A | 1 | 3.84 | 0.0001*** | 24.9 | <0.0001*** |
| B | 1 | -2.22 | 0.0064** | -14.3 | 0.0002*** |
| C | 1 | 5.01 | <0.0001*** | 32.3 | <0.0001*** |
| D | 1 | 1.69 | 0.0269* | 19.4 | <0.0001*** |
| AB | 1 | -0.11 | 0.898 ^{NS} | -0.62 | 0.8491 ^{NS} |
| AC | 1 | -0.39 | 0.6365 ^{NS} | -2.6 | 0.4308 ^{NS} |
| AD | 1 | 0.069 | 0.9339 ^{NS} | 0.63 | 0.8491 ^{NS} |
| BC | 1 | 0.42 | 0.6155 ^{NS} | 2.6 | 0.4308 ^{NS} |
| BD | 1 | -0.39 | 0.6365 ^{NS} | -2.4 | 0.4748 ^{NS} |
| CD | 1 | -0.16 | 0.8506 ^{NS} | -1.1 | 0.7326 ^{NS} |
| A ² | 1 | -0.12 | 0.8801 ^{NS} | 1.4 | 0.6632 ^{NS} |
| B ² | 1 | -0.045 | 0.955 ^{NS} | 1.9 | 0.5541 ^{NS} |
| C ² | 1 | -0.68 | 0.3979 ^{NS} | -2.2 | 0.4793 ^{NS} |
| D ² | 1 | 0.42 | 0.6009 ^{NS} | -7.9 | 0.0264 ^{NS} |
| Residual | 11 | | | | |
| Lack of fit | 10 | | 0.129 ^{NS} | | 0.2025 ^{NS} |
| Pure error | 1 | | | | |
| Cor total | 25 | | | | |

¹⁾Designated as A for alcohol concentration (%), B for pH, C for storage temperature (°C), and D for storage time (d).

²⁾ $R^2=0.9102$; adjusted $R^2=0.796$; predicted $R^2=0.4834$; adequate precision=10.367; coefficient of variation (%)=4.63.

³⁾ $R^2=0.9688$; adjusted $R^2=0.9291$; predicted $R^2=0.8207$; adequate precision=18.648; coefficient of variation (%)=2.86.

Significantly different at * $P<0.05$, ** $P<0.01$, and *** $P<0.001$.
df, degree of freedom; Prob, probability; NS, not significant.

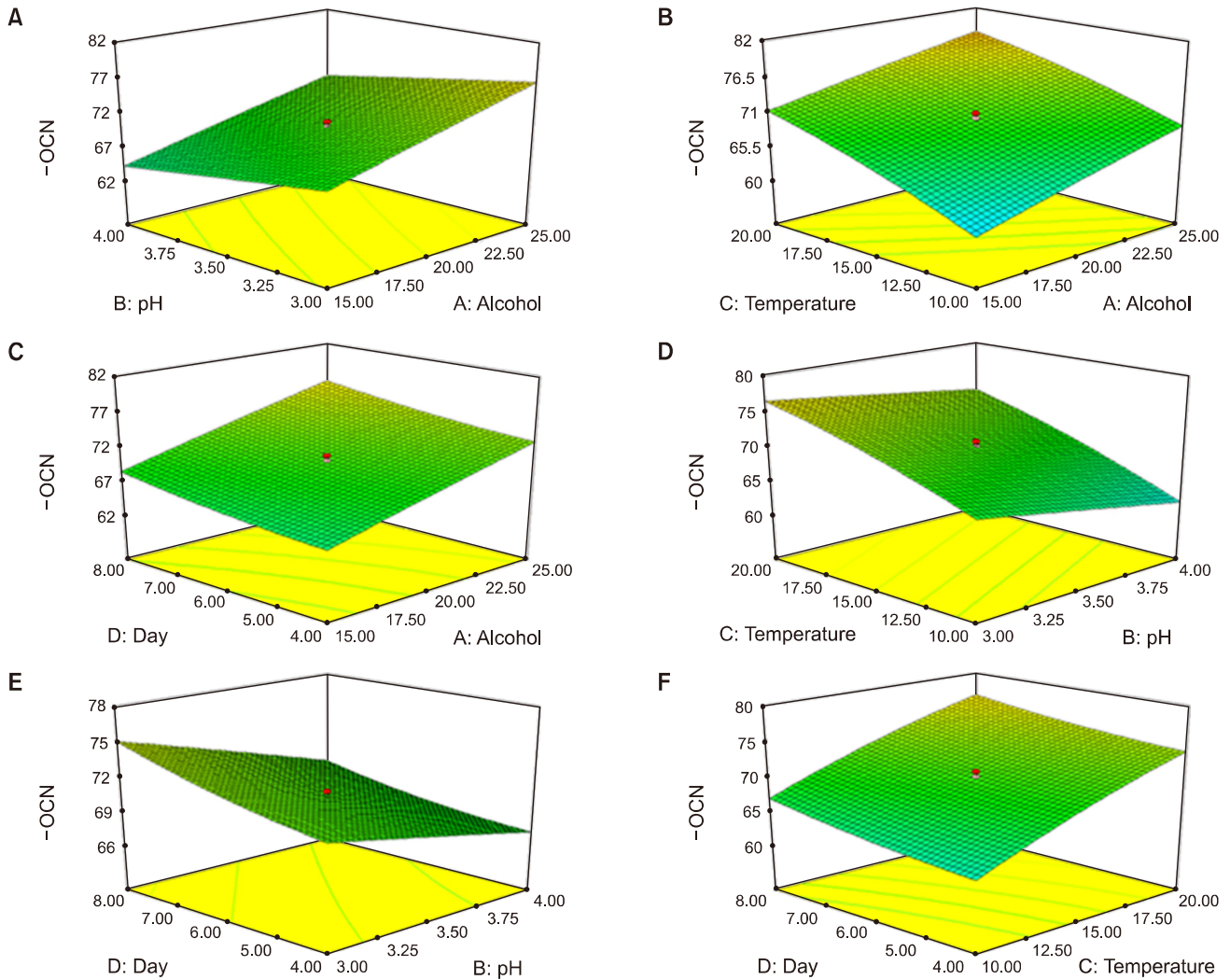


Fig. 1. Three-dimensional response surface plots depicting the interactions between alcohol concentration and pH (A), alcohol concentration and temperature (B), alcohol concentration and duration (C), pH and temperature (D), pH and duration (E), and temperature and duration (F) on cyanate degradation. The other two components (temperature, 15°C; alcohol concentration, 20%; storage duration, 6 days; pH, 3.5) are adjusted to their center values in each plot.

tion, are represented by A, B, C, and D, respectively. As shown in equation (1), the values of A, B, C, and D were significant ($P < 0.05$).

The effects of alcohol content and acidity (decreased pH) are shown in Fig. 1A. As alcohol concentration increased, cyanate degradation and acidity increased; when the alcohol concentration and pH were approximately 25% and 3, respectively, cyanate degradation was at its peak. Cyanate decomposition was affected by the concentration of alcohol used; cyanate degradation was $62.7 \pm 0.1\%$, $70.8 \pm 0.3\%$, and $81.3 \pm 0.3\%$ with alcohol concentrations of 10, 20, and 30%, respectively. Compared with the other two alcohol concentrations, 30% alcohol content was associated with higher cyanate degradation. A linear association was detected between alcohol concentration and cyanate degradation. As shown in Fig. 1B and 1C, cyanate degradation followed a similar pattern as that described by Budaev et al. (2014) and Galinaro et al. (2015). The effects of storage duration and alcohol

concentration on cyanate degradation are shown in Fig. 1C. Cyanate degradation was $64.3 \pm 0.2\%$ and $70.8 \pm 0.3\%$ at 2 and 6 days, respectively, i.e., the longer the storage duration, the higher the level of cyanate degradation. Fig. 1E and 1F show that a similar trend existed for the association between storage temperature and cyanate degradation. The effects of pH and storage temperature on cyanate degradation are shown in Fig. 1D. During cyanate degradation, the acidity of the solution increased as the storage temperature increased. When the model solutions were analyzed at different pH levels, i.e., 2.5, 3.0, 3.5, 4.0, and 4.5, cyanate degradation was slightly increased with lower pH solutions, i.e., under acidic conditions. Cyanate degradation followed a similar pattern as that shown in Fig. 1A and 1E. Fig. 1F depicts the interaction effects of storage temperature and duration on cyanate degradation, which increased as storage temperature and duration increased. The effect of storage temperature on cyanate degradation was significant. Furthermore, as shown in Fig.

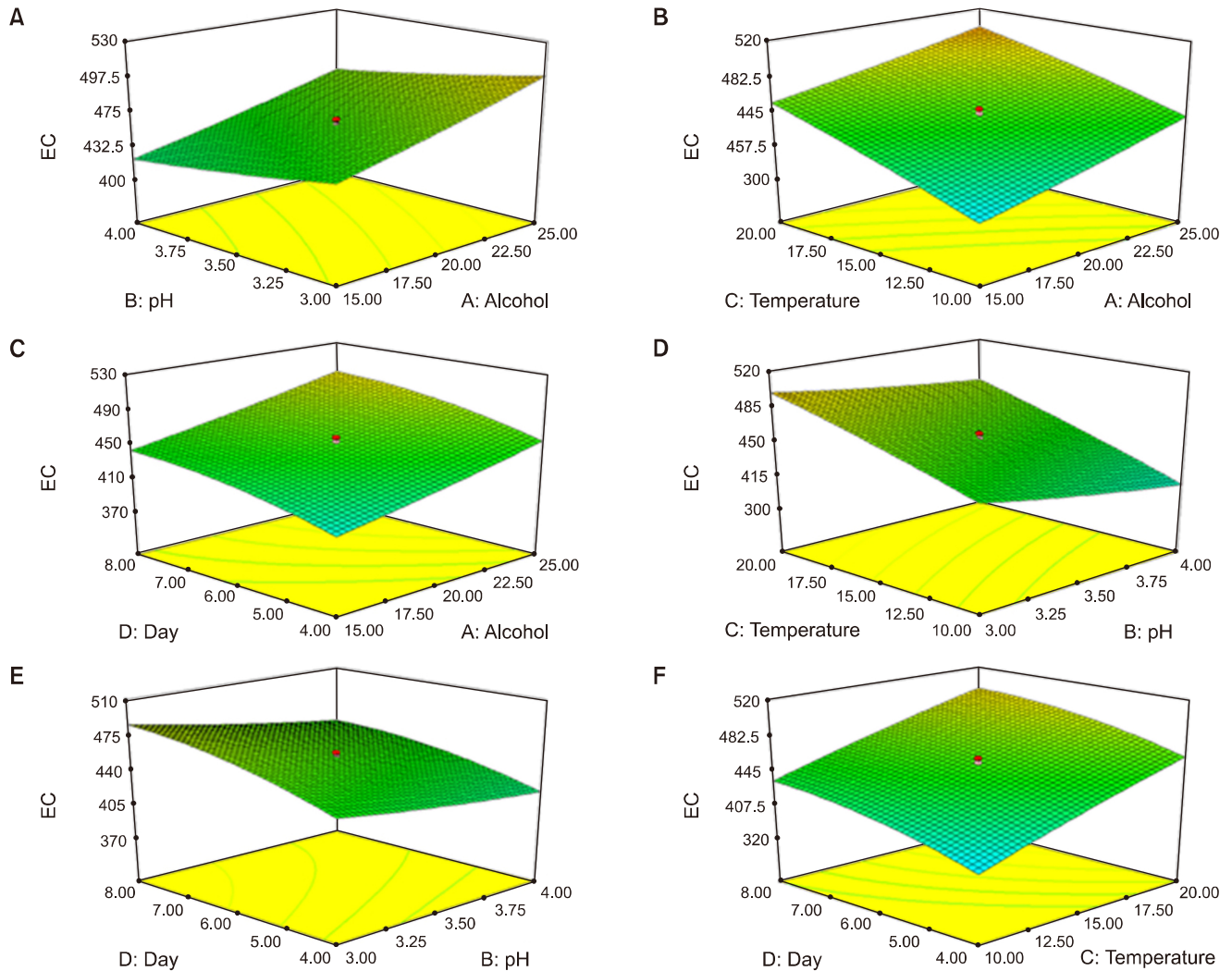


Fig. 2. Three-dimensional response surface plots demonstrating the interaction of alcohol concentration and pH (A), alcohol concentration and temperature (B), alcohol concentration and duration (C), pH and temperature (D), pH and duration (E), and temperature and duration (F) on ethyl carbamate (EC) formation. The other two components (temperature, 15°C; alcohol concentration, 20%; storage duration, 6 days; and pH, 3.5) are fixed to their central levels in each plot.

1B and 1D, cyanate degradation increased as storage temperature increased. In addition, a linear relationship existed between cyanate degradation and storage temperature, suggesting that this reaction was temperature-sensitive. When the storage temperature of the solution was 25°C, cyanate degradation was $79.4 \pm 0.2\%$; when the temperature was reduced to 5°C, cyanate degradation was $60.1 \pm 0.1\%$. Thus, cyanate degradation under 25°C and 5°C conditions differed significantly, confirming that temperature had an impact on cyanate decomposition.

EC formation

As shown in Table 3, EC content values derived using the response surface quadratic model were positively linked ($R^2=0.9688$). The sufficient accuracy value, a signal-to-noise ratio index for the model, was 18.648. These results showed that the model could be used to study the system design. The F-value, 24.40, indicates that the EC values produced using this approach for samples stored

at different alcohol conditions, temperature, pH, and storage duration were highly significant ($P < 0.05$), i.e., the processing methods had a significant impact on EC formation. Despite a strong positive coefficient link ($R^2 = 0.97$) and low coefficient of variation ($CV = 2.86$), the lack of fit F-value of 14.40 showed that the lack of fit was not significant relative to the pure error. There was a 20.25% chance that a high lack of fit F-value would occur due to noise. When the lack of fit is not significant, the model fit the data.

$$EC = 455.5 + 24.9A - 14.3B + 32.3C + 19.4D - 0.62AB - 2.6AC + 0.63AD + 2.6BC - 2.4BD - 1.1CD + 1.4A^2 + 1.9B^2 - 2.2C^2 - 7.9D^2 \quad (2)$$

Equation (2) can be used to provide assumptions regarding the formation of EC for a given set of variables. Indeed, it can be used to determine the relative impact of the variables by evaluating the factor coefficients of the

estimates given in Table 3. The values of A, B, C, and D were significant at $P < 0.05$, as stated in equation (2).

The interaction of alcohol concentration and acidity in the production of EC is described in Fig. 2A. The amount of alcohol in the mixture affected the formation of EC from the reaction of ethanol and cyanate. EC production was linearly related to the amount of alcohol consumed. Fig. 2B and 2C show the same pattern. EC was formed at $406 \pm 5 \mu\text{M}$ and $458 \pm 4 \mu\text{M}$ when the alcohol concentrations were 10% and 20%, respectively, and EC levels were significantly higher with a 20% alcohol concentration. Thus, the model solution with a higher alcoholic content had a higher rate of EC production. The effects of storage duration and alcohol content on EC formation are shown in Fig. 2C. EC levels increased as storage duration and alcohol content increased. When the other components were set to their middle values (i.e., alcohol content, 20%; pH, 3.5; temperature, 15°C), EC formation was $379 \pm 9 \mu\text{M}$ in the first 2 days of storage and $453 \pm 3 \mu\text{M}$ at 6 days. This suggests that EC content increased due to storage duration. Wu et al. (2014) previously stated that EC develops during storage. Several studies have revealed that EC levels change in alcoholic beverages with storage temperature and duration (Li et al., 2015). Additionally, limiting storage duration, lowering temperature, and avoiding light in the storage environment can all help slow down the synthesis of EC (Gowd et al., 2018). Fig. 2D shows the effects of pH and temperature on EC production. When pH was low (< 3), the reaction appeared to be stable. However, EC levels increased significantly with increases in alcohol concentration and acidity. Although the effect was not substantial, EC production clearly increased at low pH (with minimal impact) (Fig. 2D and 2E). Alberts et al. (2011) reported that EC production is enhanced in acidic media. Additionally, Kim et al. (2015) found that EC production is often enhanced in an acidic environment. Our results indicated that pH had only a minor impact on EC production. Fig. 2A and 2D both show a similar pattern.

The effects of storage temperature and duration on EC generation are shown in Fig. 2F; the generation of EC increased with the temperature during sample storage, i.e., from 5°C to 25°C . Thus, EC levels increase during storage and are significantly affected by storage temperature. Fig. 2B and 2D show a similar pattern. At temperatures of $> 15^\circ\text{C}$, the EC production rate in the model solution significantly increased. In the three model solutions with storage temperatures of 5, 15, and 25°C , the levels of EC were 389 ± 9 , 453 ± 3 , and $514 \pm 4 \mu\text{M}$, respectively; thus, the sample at 5°C had significantly less EC content than that at 15°C , and the solution at 25°C had significantly higher EC formation than that of the other two model solutions. EC is formed at various temperatures during storage. High temperatures increase EC formation

more quickly than low temperatures (Wu et al., 2012). Several other investigations have found that high temperatures and long-term storage significantly accelerate the production of EC (e.g., Wu et al., 2014). Somtochukwu et al. (2018) found that the storage duration of alcoholic beverages was proportional to their EC concentration and storage temperature. The rate of EC production increases with storage duration and shows an exponential increase at higher temperatures (European Food Safety Authority, 2007). Liu et al. (2012) also revealed that EC generation increases as temperature rises. Moreover, Zhou et al. (2021) found that EC mostly accumulates during heat treatment after fermentation rather than during fermentation itself. In addition, EC has been shown to increase during thermal treatment (Zhou et al., 2019). Ma et al. (2021) indicated that storage temperature has a substantial impact on the development of EC; similarly, Vázquez et al. (2017) found that EC levels were high at higher temperatures.

Relationship between cyanate degradation and EC formation and their variables

The relationship between cyanate degradation and EC formation was explained using equation (3), which was obtained from the data in Table 3.

$$\text{EC formation (\%)} = 6.617 \text{ cyanate degradation (\%)} - 11.499 \quad (3)$$

Equation (3) shows the linear relationship between cyanate degradation and EC formation (coefficient of determination $R^2 = 0.9775$); thus, there was a strong relationship between cyanate degradation and EC formation. Cyanate degradation increased with increasing EC formation due to autoxidation of cyanide into cyanate, which further reacts with ethanol to form EC. When $70.0 \pm 0.1\%$ of cyanate degrades to $453 \pm 3 \mu\text{M}$ EC and $83.1 \pm 0.1\%$ of cyanate degrades to $538 \pm 9 \mu\text{M}$ of EC, this implies that not all cyanate reacts with ethanol during fermentation to form EC. These results support the proposal that cyanate is an important EC precursor (Galinaro et al., 2015). The formation of EC increased gradually as storage temperature and alcoholic content increased in the storage media, whereas other parameters remained constant. The effects of pH and storage duration were inversely and directly proportional to EC formation and cyanate degradation, respectively. However, variation in these parameters during the experiments led to low or high EC and cyanate amounts. Alcohol condition and storage temperature during the experiments are suggested to be main factors that influence EC formation and cyanate degradation.

Provided that other parameters remained constant, continuously increasing alcohol content and storage tem-

perature resulted in an increase in EC formation and cyanate degradation. Therefore, the lowest level of EC formation ($348 \pm 3 \mu\text{M}$) and cyanate degradation ($53.8 \pm 0.1\%$) were obtained at a low temperature (10°C), alcohol concentration (15%, v/v), and acidic condition (pH 4) over a short storage duration (4 days). Similarly, the highest levels of EC ($538 \pm 9 \mu\text{M}$) and cyanate degradation ($83.1 \pm 0.1\%$) were obtained in samples with high alcohol content (25%, v/v), storage temperature of 20°C , and acidic condition (pH 3) over an extended experimental duration (8 days). The same results were observed during pilot experiments when the highest level of EC formation ($573 \pm 5 \mu\text{M}$) was obtained at 40°C ; this level was reduced ($412 \pm 3 \mu\text{M}$) at 20°C for analysis performed for 2 h. Moreover, appropriate storage conditions with storage at low temperature over a short period of time can effectively reduce EC formation, as reported in previous studies (Cook et al., 1990; Aresta et al., 2001; Riachi et al., 2014; Wu et al., 2014).

In the present study, the cyanate reaction in an ethanol-water media produced EC. RSM was able to optimize the conditions of EC formation. Storage temperature, duration, alcohol concentration, and pH influenced the rate of EC production. Temperature had the greatest impact on EC formation, followed by alcohol concentration, storage duration, and pH. To reduce the formation of EC in alcoholic drinks and fermented foods, storage duration should be shortened and storage temperature lowered. The conditions tested in this study provide important information about EC formation that could be used to reduce EC formation during storage. Consequently, the government must increase public awareness about EC formation in alcoholic drinks and fermented foods, and they should monitor EC content in alcoholic beverages on a regular basis. The standard for EC levels in alcoholic beverages must also be considered.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

AUTHOR DISTRIBUTIONS

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