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# Spectrophotometric determination of ethyl carbamate through bi-enzymatic cascade reactions†

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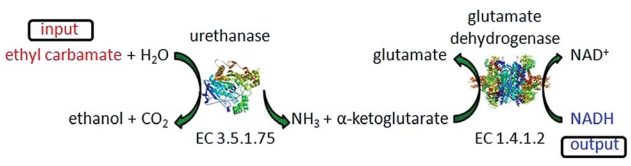
A highly sensitive spectrophotometric method for ethyl carbamate (EC) determination was established through glutamate dehydrogenase/urethanase cascade reactions and the corresponding change in NADH concentration. The absorbance at 340 nm is linearly related to the EC concentration within the range of 0.3–50 μM, with a low detection limit of 0.00928 μM. The assay was further applied to analyse EC in mimic Chinese rice wine samples.

Ethyl carbamate (EC or urethane, $C_2H_5OCONH_2$) is known as a genotoxic carcinogen[1] that widely exists in fermented foods and alcoholic beverages, such as bread, yogurt, cheese, brandy, Chinese rice wine, sake and wine,[2] due to the natural biochemical processes in the fermentation process.[3] EC was presently classified as a probable human carcinogen (Group 2A) by the World Health Organization's International Agency for Research on Cancer (IARC).[1b] The Canadian federal government and USFDA established guidelines to limit the content of EC in alcoholic beverages.[4] After that, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) further suggested that the EC content in fermented beverages and foods should be strictly reduced as far as possible.[5] Therefore, the determination and control of EC content are of significant importance.

Unfortunately, the determination of EC is not easy because of its low concentration in the samples and lack of typical physicochemical properties. So far, various methods have been developed to quantitatively analyse EC in low concentrations in alcoholic beverages and fermented foods. As early as in 1974, EC in wine was extracted with chloroform and analysed by gas-liquid chromatography.[4,6] After that, gas chromatography (GC) was widely employed in EC detection, and frequently coupled with mass spectrometry (MS).[7] In recent years, a variety of microextraction techniques were developed to pretreat the samples,[8] and different detectors were coupled with GC, such as GC-thermal energy analyser (TEA), GC-nitrogen phosphorus thermionic detector (NPD), GC-fluorescence detector (FLD), *etc.*[4,7b,9] High-performance liquid chromatography (HPLC) coupled with MS or FLD was also frequently reported to quantify EC in fermented foods and beverages.[9c,10] Although most of these methods have good sensitivity and reproducibility, the complicated sample preparation, time-consuming process and high cost restrict their applications. To establish efficient, accurate and economical methods for the detection of EC is still an important issue in food safety control.

Enzyme-based assays and the corresponding biosensors are widely used in the field of bioengineering, medicine, food, environment, *etc.*[11] Taking advantage of high sensitivity, high specificity and fast response, enzyme-based sensors are very competitive in the analysis of the complicated samples. Urethanase can catalyze the decomposition of EC and therefore has great prospects in the detection and elimination of EC in food.[12] However, this enzyme is still far from commercialization. Previously, we screened a urethanase-producing strain *Penicillium variabile* JN-A525 and obtained purified urethanase. The enzyme has good thermostability and ethanol tolerance.[13] In this study, urethanase is combined with glutamate dehydrogenase (GLDH) to fabricate a bi-enzymatic cascade reaction for spectrophotometric detection of EC.

Scheme 1 illustrates the principle of the detection. EC can be decomposed by urethanase, producing ethanol, $CO_2$ and ammonia. Then ammonia can be utilized as one of the substrates and further converted to glutamate in the presence of




Scheme 1 Schematic illustration of EC determination.

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the co-substrate α-ketoglutarate, which is catalysed by glutamate dehydrogenase. The catalysis of GLDH requires the presence of its cofactor reduced β-nicotinamide adenine dinucleotide (NADH), which is converted to the corresponding oxidized form, $NAD^+$, during the reaction. The characteristic UV-visible absorption of NADH can then be used as output, *i.e.* the absorbance at 340 nm can be used in the quantitative determination of EC.

Prior to detection, the conditions of the bi-enzymatic cascade reaction system were optimized, including the reaction pH, the concentration of urethanase and GLDH, and the concentration of the co-substrate α-ketoglutarate. Firstly, the influence of solution pH on the rate of cascade reactions was investigated. It is well known that enzymatic reactions reach the highest rate at the optimal pH of the enzymes. Unfortunately, the two enzymes in the cascade reaction system differ in their optimal pH. Urethanase, which catalyses the hydrolyzation of EC, has the optimal pH of 4.5, whereas GLDH, which catalyses the formation of glutamate and simultaneously oxidizes NADH to $NAD^+$, has the optimal pH of 8.3. Therefore, it is significant to find an intermediate pH at which both enzymes can work well to achieve the highest overall rate of the whole cascade reactions. The catalysis was carried out at different pHs in 25 mM citrate buffer (pH 4.5–5.5), phosphate buffer (pH 6.0–7.5) and Tris–HCl buffer (pH 7.5–8.0), respectively. The overall reaction rate can be expressed as the depletion rate of NADH, therefore the decrease in absorbance at 340 nm. The results are shown in Fig. 1a, the absorbance declines slowly at either pH 4.5 or pH 8.0, which is around the optimal pH of one of the enzymes. However, the absorbance decreases with the fastest rate at pH 6.0. So it can be concluded that although both of the enzymes do not achieve their individual highest reaction rate, the overall reaction rate reaches the fastest at this pH. Therefore, pH 6.0 is chosen as the optimal pH of the bi-enzymatic system.

The concentrations of urethanase and GLDH added into the reaction system were then optimized. Urethanase catalyses the first and key step of the cascade reactions. To optimize the concentration of urethanase, the concentration of GLDH was fixed at 8 U $mL^{-1}$ while the concentration of urethanase varied in the range of 4–16 U $mL^{-1}$. As shown in Fig. 1b, the change of absorbance at 340 nm increases with the increasing addition of urethanase. When the activity of urethanase reaches 16 U $mL^{-1}$, the depletion rate of NADH is greatly accelerated, which can meet the requirement of fast reaction and detection. Considering the cost of urethanase, the concentration of 16 U $mL^{-1}$ was selected. Meanwhile, the influence of GLDH concentration was also investigated with the fixed urethanase concentration of 16 U $mL^{-1}$. As shown in Fig. 1c, the depletion rate of NADH is initially accelerated with the increasing concentration of GLDH. However, when the activity is more than 10 U $mL^{-1}$, the reaction rate no longer increases, and even slightly decreases. This may be attributed to the ratio of NADH and the enzyme, and the increased reverse reaction rate. Therefore, the concentration of GLDH was set as 10 U $mL^{-1}$.

α-Ketoglutarate acts as one of the substrates of GLDH in the bi-enzymatic system. The co-existence of α-ketoglutarate is essential for the detection. Its concentration also influences the reaction rate. α-Ketoglutarate at low concentration can activate GLDH and further accelerate the oxidation rate of NADH. However, high concentration of α-ketoglutarate can inhibit the enzyme activity.[14] Then different amounts of α-ketoglutarate were added into the reaction system and the change of absorbance at 340 nm was measured. As shown in Fig. 1d, when the concentration of α-ketoglutarate is lower than 60 mM, the reaction rate increased with the increasing concentration of α-ketoglutarate. The trend reverses when α-ketoglutarate is higher than 60 mM, which can be attributed to the gradual inactivation of GLDH with high concentration of α-ketoglutarate. Thus the optimal concentration of α-ketoglutarate is 60 mM for the bi-enzyme cascade reaction.

After the optimization of experimental conditions, the detection of EC was carried out. A total of 600 μL of the reaction solution in 25 mM PBS (pH 6.0) containing 16 U $mL^{-1}$ of urethanase, 10 U $mL^{-1}$ of GLDH, 60 mM of α-ketoglutarate and 0.25 mM of NADH was established. Different concentrations of EC were added into the reaction solution, then the absorbance at 340 nm was continuously monitored, and the absorbance change during the reaction time of 5 min was calculated. As shown in Fig. 2, the change in absorbance increases with the increasing concentration of EC. Obviously, the increased concentration of EC depletes more NADH, which further leads to the decrease of absorbance at 340 nm. As the concentration of EC reaches 1 mM, the change in absorbance levels off due to the complete depletion of NADH. Furthermore, a linear relationship can be derived in the EC concentration range of 0.3–50 μM. The linear regression equation is $y = 0.00468x + 0.03329$, $R^2 = 0.990$, where $y$ represents the absorbance change during 5 min, and $x$ represents the concentration of EC (μM).

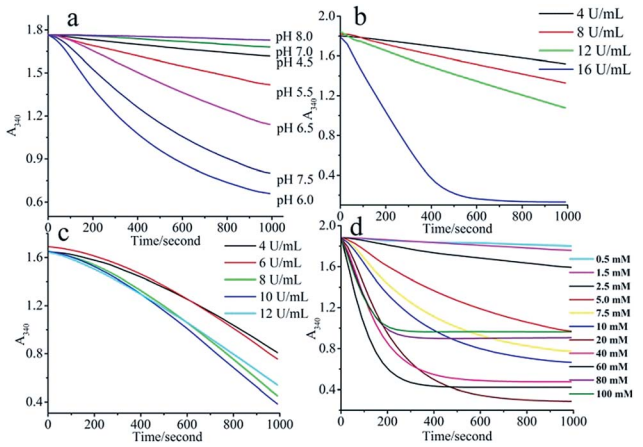


Fig. 1 (a) The influence of pH on the cascade reaction rate. Other conditions: 5.4 mM EC, 8 U $mL^{-1}$ of urethanase and GLDH, 0.25 mM α-ketoglutarate and 0.25 mM NADH; (b) the influence of urethanase concentration on the cascade reactions. Reaction conditions: pH 6.0, 8 U $mL^{-1}$ GLDH, the other conditions are the same as (a); (c) the influence of GLDH concentration on the cascade reactions. Reaction conditions: pH 6.0, 16 U $mL^{-1}$ urethanase, the other conditions are the same as (a); (d) the influence of α-ketoglutarate concentration on the cascade reactions. Reaction conditions: pH 6.0, 16 U $mL^{-1}$ urethanase, 10 U $mL^{-1}$ GLDH, 5.4 mM EC, 0.25 mM NADH.

 

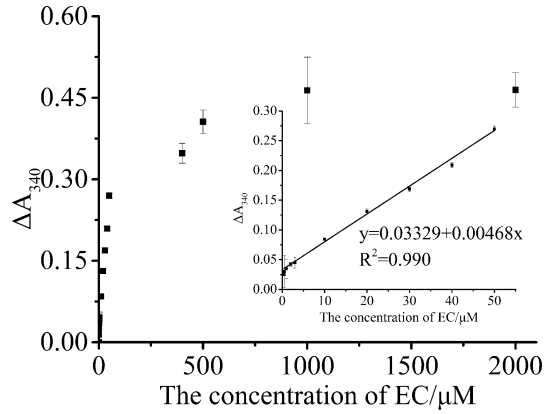


Fig. 2 The relationship between the change in absorbance at 340 nm and the concentration of EC. The inset shows the derived linear relationship and regression equation. The reaction time is 5 min.

The low detection limit of 0.00928 μM can be derived from the EC concentration at negative control + 2 × SD.

In order to examine the precision and accuracy of the spectrophotometric method for the determination of EC in real samples, a recovery test was performed by adding the EC standard solution to the synthetic Chinese rice wine samples at three specific concentrations (5, 10 and 20 μM). Each sample was analyzed four times. The results are listed in Table 1. The recovery was between 96.7% and 100.01%, indicating a high accuracy of the detection. From four independent determinations of the same samples, the relative standard deviation (RSD) of 1.634–4.611% ($n = 4$) can be achieved, showing that the proposed method has satisfactory precision.

In summary, we report a novel spectrophotometric method for EC determination based on bi-enzymatic cascade reactions. To the best of our knowledge, this is the first report of an enzyme-based detection method for EC. Compared to most frequently used methods, such as GC-MS, HPLC-FLD, HPLC/MS/MS, and MEPS/GC-MS, the established method does not need an expensive instrument. The specificity of the method can be ensured by urethanase, which catalyzes the direct conversion of EC. According to our previous substrate specificity study, urethanase used in this work shows weak activity towards methyl carbamate, and negligible activities towards other substrates such as glutamic acid, γ-aminobutyric acid and glycine.[13a] Due to the high specificity and sensitivity of the two enzymes towards their substrates, the assay can be carried out without the pretreatment of the samples, such as organic solvent extraction, thus it can achieve simple, fast and accurate detection of EC. Under the optimized conditions, a linear detection range of 0.3–50 μM with a detection limit of 0.00928 μM can be achieved, which are in accordance with the concentration of EC in fermented beverages. The sensitivity of this assay is similar to some reported methods, such as HPLC-FLD, GC-MS, *etc.*[9e,10b,c] Although more sensitive methods have also been reported, this assay is superior in the aspects like pretreatment-free and solvent-free conditions, fast detection and low-cost. Therefore, the established assay may have great prospects in EC determination and control.

Table 1 The recovery and RSD of the spectrophotometric assay for real samples

| Added (μM) | Found (μM) | Recovery (%) | RSD (%) |
|---|---|---|---|
| 5 | 4.8353 | 96.71 | 4.611 |
| 10 | 9.5535 | 95.54 | 2.35 |
| 20 | 20.0032 | 100.01 | 1.634 |

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