Occurrence of ethyl carbamate in three types of Chinese wines and its possible reasons

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Abstract A total of 75 wines including 30 white wines, 31 red wines, and 14 sparkling wines were obtained from several regions in China (Sinkiang, Tonghua, Huailai, Yantai, Changli, Shanxi, Gansu, and Ningxia). Ethyl carbamate (EC) was detected by gas chromatography mass spectrometry. The EC concentration ranged from less than 1.16 to 38.56 µg/L, and the concentrations in 17 wines exceeded the U.S. limit for table wines (15 µg/L). The concentrations of EC increased in the order of white, red, and sparkling wines with the corresponding mean concentrations of 6.12, 9.22, and 14.03 µg/L. The relationship between EC concentration and wine type suggested that EC concentrations in wines might be affected by vinification patterns, most likely due to the difference between EC precursors in different vinification processes. This work provides a novel clue for EC contamination in different wines.

Keywords: ethyl carbamate, Chinese wine, vinification pattern, precursor, sparkling wine

Introduction

Ethyl carbamate (EC), also called urethane, was proved toxic early in 1943, and its carcinogenicity has caused worldwide public concern (1). EC was classified into group 2B (possibly carcinogenic to humans) in 1974 by the International Agency for Research on Cancer and was reclassified into group 2A (probable carcinogenic to humans) in 2007 (2) owing to the confirmed correlation between EC and vascular liver tumor in human beings (3).

EC is a contaminant naturally accumulated during fermentation or storage of alcoholic beverages and fermented foods. The concentration scale of EC in these foods ranges from ng/L to mg/L (4-6). EC was first detected in alcoholic beverages, the main source of public EC intake in 1976 (7). Moreover, with a growing public concern about food safety, EC in alcohol beverages attracts growing attention. Several alcoholic beverages (8) including distilled spirit (6), yellow rice wine (9), sake (10), and wine (11) had been found with detectable levels of EC. The EC concentration in some beverages is up to more than 100 mg/L, while the benchmark dose lower limit is only 0.3 mg/kg per day (2). Therefore, it is critically important to control and restrict EC contamination in alcoholic beverages.

The mandatory law about EC levels in alcoholic beverages was first passed in Canada in 1985, stating that the legal limits of table wine, fortified wine, distilled spirit, and fruit brandy are 30, 100, 150, and 400 µg/L, respectively (12). In 1988, U.S. Food and Drug Administration

(FDA) accepted a plan to reduce EC levels in wine. The upper limits of EC in table wines (alcohol content less than 14% v/v) produced after 1988 and dessert wines (alcohol content more than 14% v/v) produced after 1989 were 15 and 60 µg/L, respectively (5). No rule or regulation on the EC limit in alcoholic beverages has been put into force in China.

The formation of EC in wine has been investigated extensively. The primary precursors of EC in wines are urea, citrulline, and carbamyl phosphate (7,13,14). Moreover, compounds with carbamyl groups such as purine and pyrimidine can also be transformed into EC (15). EC is produced in a spontaneous chemical reaction involving ethanol and its precursors. The synthesis could continue as long as the precursors exist. Thus, the concentration of EC in wines is modestly dependent on that of EC precursors to some extent. Wines with a higher level of EC precursors are more inclined to form EC. These precursors are mainly produced by microorganisms including yeast and lactic acid bacteria (LAB), which are the major microorganisms contributing to ethanol and malolactic fermentation, respectively (16). Therefore, it was hypothesized that vinification patterns may make a significant impact on EC concentrations by regulating the EC precursors in different types of wines.

Numerous studies on the occurrence of EC in different types of wines including red wines, white wines, and brandy were carried out in many countries (8,11,17). But a general investigation of EC occurrence was conducted without focusing on the distinction among different

types of wine. Alberts (18) reported a significant difference of EC concentrations in red wines, white wines, brandy, and fortified wines, but the factor resulting in the difference was not analyzed further. Therefore, reports on distinction of EC concentrations in different types of wines are lacking. Meanwhile, as an important role of wines, EC contamination in sparkling wines deserves more attention. Consequently, the current study aimed to detect EC in Chinese wines including white, red, and sparkling wines and verify whether there is a correlation between vinification patterns and EC concentrations in different type of wines.

Materials and Methods

Wine samples A total of 75 wines including white wines ($n=30$), red wines ($n=31$), and sparkling wines ($n=14$) were collected from winemaking regions in China (Sinkiang, Tonghua, Huailai, Yantai, Changli, Shanxi, Gansu, and Ningxia).

Reagents and chemicals EC (purity >99%) and *n*-propyl carbamate (*n*-PC, purity >98%) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethanol (gas chromatography grade) was purchased from Merck (Darmstadt, Germany). Anhydrous sodium sulfate, dichloromethane, and ammonium sulfate are of analytical grade and were obtained from Beijing Chemical Works (Beijing, China). Water was purified through a Milli-Q System (Merck Milipore, Molsheim, France).

Sample preparation Liquid/liquid extraction of EC was carried out by the method described by Ough (7) with some modifications. The details are as follows: 10 μ L of *n*-PC (100 μ g/mL in ethanol) was added into 20 mL of wine as internal standard. Then 6 g of ammonium sulfate was added to increase the ionic strength of the wine and reduce solubilization. The wine were then added with 10, 5, and 5 mL of dichloromethane. The organic fractions were obtained after stirring with a vortex mixer (1 min) and centrifuging at 4°C (16,000 $\times g$, 15 min), consecutively. The organic fractions were combined and centrifuged at 4°C (16,000 $\times g$, 15 min) to break the emulsion. The extracts were then dried with anhydrous sodium sulfate and concentrated in a rotary evaporator to a final volume of 3–4 mL. Finally, the extracts were concentrated to 1 mL under a mild stream of pure nitrogen gas. The concentrate was transferred to a vial, and then sealed and stored at 20°C prior to GC analysis. Each sample was extracted in triplicate.

Gas chromatography mass spectrometry (GC-MS) An Agilent 6890N GC (Agilent Technologies, Santa Clara, CA, USA), equipped with an Agilent 5973 MS (Agilent Technologies), was used for detection and quantification of EC. An INNOWAX capillary column (30 m $\times$ 0.25 mm i.d.; film thickness 0.25 μ m) (J&W Scientific, Folsom, CA, USA) was used for chromatographic separations. The carrier gas was helium (1 mL/min). The GC oven was programmed at an initial temperature

of 50°C and then ramped up at 7°C/min to 110°C, at 3°C/min to 150°C, and at 15°C/min again to 220°C, followed by a 2 min hold. The injector temperature was 200°C and the detector temperature was 250°C. The ion source temperature was 230°C. The electron energy was 70 eV. Data were acquired in selected ion monitoring mode, and the selected ions were *m/z* 62, 74, and 89. The quantitative ion was *m/z* 62. Analyte of 1 μ L was injected in splitless mode. The solvent delay was 7 min. The compounds were identified by comparing their retention time and mass spectra with those of pure reference standards.

Results and Discussion

Method validation A calibration curve comprised of six standard solutions was established. The squared correlation coefficient (r^2) was 0.995, indicating a strong linear relationship. As for sensitivity, the limit of detection (LOD) and the limit of quantification (LOQ) were calculated with signal-to-noise ratio (S/N) of 3 and 10, respectively. The low LOD and LOQ (1.16 and 3.86 μ g/L, respectively) were close to or even smaller than those reported in literatures (19–21), which demonstrated the high sensitivity of this methodology in the detection of EC in wines. The accuracy was evaluated by recovery, which was calculated on the basis of concentration variation by adding four known amounts of EC to wines. The recoveries of this method were between 94.7 and 107.4%, which confirmed its high accuracy. Therefore, with high linearity, sensitivity, and accuracy, the method was suitable for detection of EC in wines. Method validation data are presented in Table 1.

Detection of EC in Chinese wines The validated method was used to detect EC in three types of wines including red, white, and sparkling wines from China. Figure 1 shows a GC chromatogram of a wine sample. The general situation of EC contamination in Chinese

Table 1. Analytical method validation data

Validation parameter		Result	
Calibration curve		$Y^1=1.6424X^2+0.001$	
Linear range (μ g/L)		5–150	
r^2		0.995	
LOD (μ g/L) ³⁾		1.16	
LOQ (μ g/L) ⁴⁾		3.86	
Fortification (μ g/L)	Recovery (%)	RSD _i (%) ⁵⁾	RSD _R (%) ⁶⁾
2	101.1	1.41	5.32
5	107.4	2.79	6.77
10	94.7	1.45	2.74
50	98.2	0.76	3.35

¹⁾Y: area ratio between EC and *n*-PC

²⁾X: concentration ratio between EC and *n*-PC

³⁾LOD (μ g/L): limit of detection

⁴⁾LOQ (μ g/L): limit of quantification

⁵⁾RSD_i (%): intra-day precision

⁶⁾RSD_R (%): inter-day precision carried out on three different days

Table 2. Ethyl carbamate (EC) contamination and frequency of occurrence in three types of wines

Wine types	No. of positive/total samples (%)	EC concentration ($\mu\text{g/L}$)			No. of samples in the range ($\mu\text{g/L}$)			
		Mean	Median	Range	<LOQ ¹⁾	LOQ-15	15-30	>30
Sparkling	14/14 (100%)	14.03	13.96	5.29-21.96	0	7	7	0
Red	29/30 (96.7%)	9.22	6.20	n.d. ²⁾ -38.56	5	19	5	1
White	27/31 (87.1%)	6.12	3.28	n.d.-24.35	16	11	4	0
Total	68/75 (90.7%)	8.84	5.67	n.d.-38.56	21	37	16	1

¹⁾Limit of quantification (LOQ) is 3.86 $\mu\text{g/L}$ ²⁾n.d. means concentration below limit of detection (LOD), calculated as LOD/2 (0.058 $\mu\text{g/L}$)**Table 3.** Ethyl carbamate (EC) contamination in wines from other countries

Region	No. of positive/total samples (ratio)	Mean ($\mu\text{g/L}$)	Range ($\mu\text{g/L}$)	No. >15 $\mu\text{g/L}$ (ratio)	No. >30 $\mu\text{g/L}$ (ratio)	Reference
Spain	35/36 (97.2%)	10.43	n.d. ¹⁾ -24.7	7 (19.4%)	-	11
Hungary	33/33 (100%)	17.7	4.9-39.9	18 (54.5%)	2 (6.1%)	17
South Africa	72/72 (100%)	11	2.4-31	13 (18.1%)	2 (2.8%)	18

¹⁾n.d. means concentration below limit of detection (LOD), calculated as LOD/2 (0.058 $\mu\text{g/L}$)

wines is presented in Table 2.

About 68 (90.7%) wine samples were EC positive (EC concentration is the mean of multiple samples), implying that EC contamination is widespread in Chinese wines. EC concentration ranges from undetectable levels to 38.56 $\mu\text{g/L}$, with the overall mean of 8.84 $\mu\text{g/L}$ when the concentration of under LOD was calculated as LOD/2 (0.58 $\mu\text{g/L}$). Thirty-three samples (44%) were at a low EC level (<5 $\mu\text{g/L}$) and 26 samples (34.7%) were between 5 and 15 $\mu\text{g/L}$. It is noteworthy that 17 wines exceeded the FDA limit of 15 $\mu\text{g/L}$ for EC in table wines, and one wine even exceeded the mandatory limit of 30 $\mu\text{g/L}$ in Canada. The over-limit ratio of EC is up to 22.7% according to the FDA limitation, indicating that EC contamination in Chinese wines is a common phenomenon.

Numerous studies on the occurrence of EC in wines were carried out all over the world, and the results are presented in Table 3. The mean of EC concentration, positive rate and over-limit ratio of 30 $\mu\text{g/L}$ in the present study were lower than those in Spain, Hungary, and South Africa. As to the over-limit ratio of 15 $\mu\text{g/L}$, it was close to that in Spain and South Africa, and fell far below that in Hungary. Although EC contamination in Chinese wines was not very serious comparatively, it should not be ignored nonetheless. The establishment of a regulation on EC limit is urgently needed in China.

Occurrence of EC in three types of Chinese wines Table 2 briefly summarizes the EC concentrations in the three types of wines. The results show that EC concentrations increased in the order of white, red, and sparkling wines with the corresponding mean concentration of 6.12, 9.22 and 14.03 $\mu\text{g/L}$. The corresponding positive rate (87.1, 96.7, and 100%), median concentration (3.28, 6.20, and 13.96 $\mu\text{g/L}$), and over-limit ratio of 15 $\mu\text{g/L}$ (12.9, 20.0, and 50.0%) also conformed to this order. The results implied that there might be an objective law that the EC contamination is intensified from white wines through red wines to sparkling wines. it was speculated that the difference

Fig. 1. GC chromatogram of a wine sample: selected ions at 62 m/z

might be attributed to the vinification patterns.

EC synthesis between EC precursors and ethanol occurs slowly in wines, while EC precursors can be converted to EC continuously during the whole vinification process. Therefore, the concentration of EC in wine depends modestly on EC precursors, which are mainly produced during the fermentation period of vinification (11). Consequently, the vinification patterns probably affected the final EC concentration by affecting EC precursors.

EC contamination in white and red wines According to the present results, the higher EC mean concentration, median concentration, positive rate, and over-limit ratio of 15 $\mu\text{g/L}$ in red wines than in white wines indicated that the former contained more EC than the latter. The trend also agrees with another report (11). It might be attributed to the difference in vinification patterns. Specifically, ethanol fermentation by yeasts is the only essential step in the vinification of most white wines, and the subsequent malolactic fermentation by LAB is necessary for most red wines.

During ethanol fermentation, urea is the only precursor of EC (7). Hence, EC concentration in this procedure is mainly affected by urea concentration. Urea primarily comes from arginine, the most abundant

Fig. 2. Formation of ethyl carbamate (EC) in ethanol fermentation. Arginine could be converted to urea by arginase in yeasts, followed by the reaction between ethanol and urea to form EC. Nitrogen catabolite repression (NCR) leads to inhibition of arginine utilization resulting in decline of urea synthesis, which is followed by a decrease of EC content in white wines

amino acid in grapes. Under a certain condition, after generation, urea would be immediately transformed by yeasts into ammonia and carbon dioxide (22). However, as urea is a poor nitrogen source, yeasts would select other adaptable nitrogen sources (e.g., ammonia, glutamine, and asparagine) to maintain efficient growth; this regulation mechanism is called nitrogen catabolite repression (NCR, Fig. 2) (23,24). Thus, yeasts would utilize other nitrogen sources prior to urea. As a result, urea would be accumulated in cells and released to the surroundings. EC was the product formed by urea and ethanol. Similar to urea, arginine is also “poor nitrogen” compared with “good nitrogen” such as ammonium. Therefore, arginine is strongly repressed by NCR and hardly utilized in the presence of inorganic nitrogen, leading to the decline of urea synthesis and thereby to the reduction of EC levels (22,25). Consequently, although urea was produced in ethanol fermentation, the small amount did not induce a high risk of EC contamination.

Malolactic fermentation usually proceeds once alcoholic fermentation is completed in red wines. During malolactic fermentation, most LAB including *Leuconostoc*, *Lactobacillus*, and *Pediococcus* can break arginine down to two EC precursors (citrulline and carbamoyl phosphate) via the arginine deiminase (ADI, Fig. 3) pathway (26–28). Since arginine is a major amino acid in grapes, some residues were produced at the end of ethanol fermentation. In the ADI pathway of wine LAB, residual arginine was converted to citrulline, and then the latter was disintegrated to ornithine and carbamoyl phosphate (27). Citrulline as an intermediate product is also excreted during ADI (29,30). About 4–28% of arginine could be excreted as citrulline, mainly because citrulline formation was faster than its disintegration (26,27). The citrulline, ornithine, and carbamoyl phosphate produced in the malolactic fermentation are potential EC precursors, which would result in a high risk of EC contamination in red wines (11).

Moreover, only one precursor, urea, was produced in the vinification of white wines, while three precursors, urea, carbamoyl phosphate, and citrulline, were produced in red wines. Consequently, white wines imposed a lower risk of EC contamination than red wines.

In addition, the temperature of ethanol fermentation also plays an important role in the EC synthesis. The fermentation temperature of red wines is 26–30°C, while that of white wines is 18–20°C. Previous

Fig. 3. Formation of ethyl carbamate (EC) in malolactic fermentation. In the arginine deiminase (ADI) pathway, arginine could be disintegrated into citrulline and carbamoyl phosphate, which are important EC precursors

work confirmed that the formation of EC via urea was enhanced significantly at higher temperature. As reported, the formation rates of EC via urea varied with temperature, which were 0.0004, 0.0017, and 0.0061 µg EC per mg urea per day at 13.3, 18.6, and 23.9°C, respectively (14). It also agreed with other results (31) that the formation rates increased twofold with an increase of 11°C. The higher temperature during the red wine production would be an important factor resulting in higher EC concentration.

In conclusion, red wines are more likely to generate EC, most probably owing to the high contents of EC precursors and the higher fermentation temperature. The more diverse precursors and higher fermentation temperature in red wines would lead to a higher risk of EC contamination relative to white wines.

EC contamination in sparkling wines No sparkling wines were at low EC concentrations (<LOQ), while 7 wines (50%) exceeded the FAD limit (15 µg/L) for table wines, which suggests the high risk of EC contamination in sparkling wines. This might be attributed to the special vinification process of sparkling wine.

There are two successive fermentation processes in the vinification of sparkling wines. The first one transforms grape must into base wine, while the second one occurs at a specific condition of high pressure and high ethanol content in bottles or tanks (32). It is in the second process that yeast autolysis occurs. In this process, yeasts would self-destruct and release several types of organic nitrogen sources (e.g., amino acids and nucleotides) into sparkling wines (33), among which arginine and purine could be converted into urea. Consequently, although there is only one EC precursor, its high concentration caused by yeast autolysis might contribute to the high risk of EC contamination in sparkling wines.

It has been confirmed that the amount of amino acids increased during yeast autolysis in sparkling wines. As reported, there was a marked change in the amounts of total amino acids as well as free arginine during the second fermentation process (34). To be specific, in the early stage of second fermentation (20–40 days), the amount of arginine remarkably declined, which suggests there was less “good nitrogen” such as ammonia, and the yeasts were starved of nitrogen and had to consume partial organic nitrogen sources such as arginine. Then yeast autolysis occurred (at 40 days), followed by the release of

arginine. In this period, there were still at least one half of viable yeasts and they survived until 90 days. That is, for a long time (at least 50 days), the viable yeasts starved of nitrogen were in the surroundings with increasing accumulation of arginine. There were more opportunities for arginine to generate an EC precursor without the regulation of NCR. Arginine can be converted to urea without any "block" from "good nitrogen," which might be the major reason why sparkling wines contained the highest EC content.

Nucleotides released from yeast autolysis might also make some difference. Some nucleotide catabolites such as purines (mg/L grade, e.g., xanthine, hypoxanthine, and uric acid) were formed and their concentrations increased during fermentation (35). As stated above, purines are important EC precursors because their structures contain carbamyl groups. The increased contents of purines might be a factor causing the high risk of EC contamination in sparkling wines.

In conclusion, compared with white and red wines, sparkling wines imposed a higher risk of EC contamination, most likely because more EC precursors were produced during yeast autolysis. Since EC contamination in sparkling wines have been scarcely studied, the analysis on EC formation and accumulation is only a speculation; further studies concerning the quantification of EC and EC precursors are needed to verify the mechanism of EC formation in sparkling wines.

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