Formation of Ethyl Carbamate in Goji wines: Effect of Goji Fruit Composition

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Abstract Ethyl carbamate (EC) is a multisite carcinogen widely occurring in alcoholic beverages. In this investigation, solid-phase extraction combined with gas chromatography mass spectrometry was employed to determine EC contents during the fermentation and storage processes, and the effects of Goji varieties on its formation were also examined. The results indicated that natural and potential EC contents were significantly affected by the varied composition of Goji fruits. The analysis of chemical properties showed differences in hundred-grain weight, water contents, amino acids, and nitrogen-to-carbon ratio for Goji berries. Citrulline was completely degraded although it is routinely considered as a non-preferred nitrogen for yeasts. Due to compositional differences, Goji wines accumulated distinct urea levels that positively correlated with the potential EC contents. Furthermore, the temperature in both the production processes highly influenced EC formation. These results contribute to a more comprehensive understanding of EC formation, and in turn, controlling EC in the Goji wine matrix.

Keywords: ethyl carbamate, Goji wines, potential contents, composition, amino acids

Introduction

Goji, also called Chinese wolfberry, has two close species including *Lycium barbarum* and *Lycium chinense*, which belong to a defoliated shrubbery, principally growing in China and other Asian countries (1). Its ripe fruits have been traditionally used as herbal medicine and functional foods in Asian countries such as Korea and Japan. Several studies have confirmed that active compounds such as polysaccharides, carotenoids, and flavonoids in Goji berries provide health-promoting effects for antiaging, vision, kidney, and liver functions (2). Goji wines have obtained increasing attention recently; they are consumed as a functional alcoholic beverage and produced by an inoculated fermentation technique. Compared with soft alcoholic drinks containing useful ingredients extracted from Goji berries (1), one of the major advantages of fermented Goji wines is that the metabolic activities and powerful enzymatic capabilities of microbes endow distinctive aroma and taste features associated with raw materials. Similar to general wine fermentation, the yeast *Saccharomyces cerevisiae* is usually employed to initiate alcohol fermentation for Goji wines. Nevertheless, the production of alcoholic beverages is often accompanied with the accumulation of undesirable components. For example, Kuang *et al.* (3) investigated the occurrence of ochratoxin A in Goji wines, which is a type of mycotoxin. It has also been noted that ethyl

carbamate (EC) is widely found in alcoholic beverages as a multisite carcinogen, but there is little information referring to the content distribution and specific formation pathways of EC for Goji wines to our best knowledge so far.

EC, also known as urethane, is a carcinogen that was reclassified into group 2A by the International Agency for Research on Cancer in 2007. Recent evidence has shown that frequent exposure to this compound may cause genetic carcinogenicity and lung tumorigenesis in animals and humans (4). Meanwhile, substantial surveys also suggest that EC ubiquitously occurs in alcoholic beverages such as grape wines, stone fruit spirits, Chinese yellow wines, and sugar cane spirits, with levels ranging from $\mu\text{g}/\text{kg}$ to mg/kg (5–8). Because the reaction of alcohol with urea, citrulline, and cyanide, which are the major precursors of EC in alcoholic beverages, can lead to EC production by non-enzymatic reactions during the fermentation and storage processes (9,10), the potential to cause EC accumulation may also exist in Goji wines. The major pathway for EC production often varies according to the types of alcoholic beverages, and various influence factors such as urea, pH, and even lignin are linked to EC formation in different beverages (11–13). Furthermore, urea metabolisms of yeasts are highly affected by the categories and contents of amino acids (14), which are often derived from raw materials used for fermentation. However, little literature has been concentrated on

the effects of raw material composition on EC formation.

Therefore, in the current study, lab-scale alcohol fermentation using different Goji fruits as raw materials was performed. The changes in EC and EC-related indices during the fermentation and storage processes were examined in order to clarify the specific EC formation pathways in Goji wines and to evaluate the effects of chemical composition of Goji fruits on EC formation.

Materials and Methods

Materials, chemicals, and strain Diatomaceous solid-phase extraction (SPE) columns (ChemElut; Agilent Technologies, Santa Clara, CA, USA) were used for the cleanup and concentration of sample solutions prior to gas chromatography mass spectrometry (GC-MS) analysis. Standard EC and *n*-propyl carbamate (*n*-PC) were purchased from Dr. Ehrenstorfer Co. (Augsburg, Germany). All other chemicals used were of analytical-reagent grade unless otherwise stated.

Saccharomyces cerevisiae 2.178 was obtained from Luzhou Wine-making Science Institute (Luzhou, China). Yeast cells were grown in malt extract medium at 28°C until the early logarithmic phase as a starter for Goji wine fermentation.

Goji wines brewing process Four dried Goji berries were employed as raw materials for fermentation and labeled as #1-#4 based on their different origins. Goji fruits #1 and #2 were obtained from Beijing Tongrentang Co., Ltd, and Goji fruit #3 was from Huaantang Pharmacy Co., Ltd, while Goji fruit #4 was from a local supermarket in Chengdu. A Goji wine brewing trial was carried out and general manufacturing procedures were detailed as follows. Initially, Goji berries were weighed, smashed, and then mixed well with water. The homogenized mixture was kept at 85°C for 10 min in a water bath, followed by addition of pectolase, and then kept for 1 h at 37°C to hydrolyze pectin. After that, *S. cerevisiae* culture suspension was inoculated into the mixture, and the final cell concentration was approximately 10⁵ cell/mL. The fermentation was carried out in a stainless steel container conditioned in an incubator at 12°C. After the fermentation process was finished, the broth was filtered through plate and frame filterpress (Yikeyin Co. Ltd., Chengdu, China). Samples were withdrawn at intervals to analyze the changes of physicochemical characteristics during the brewing process.

Evaluation of physiochemical properties of Goji fruits Assay of total sugar contents were carried out based on Zheng *et al.* (15). Polysaccharide was measured by the phenol-sulfuric acid method using glucose as the standard, which was according to the published method with some modification (16). Ethanol (95%, v/v) was used to precipitate crude polysaccharide. The precipitate was centrifuged at 7,050×g at 4°C for 10 min and the supernatant was discarded. The precipitated polysaccharide was washed out three times. The dried polysaccharide was colorimetrically measured using a UV-VIS

spectrophotometer (LAMBDA25; PerkinElmer, Waltham, MA, USA). The measurements of other physiochemical indices such as water contents, hundred-grain weight (HGW), total nitrogen, and ash contents were according to the method of China National Standard GB/T 18672-2002 for dried Goji berry.

Analysis of Goji fermentation process The enumeration of yeast cells was carried out based on the previous protocol (17). The pHs of the samples were directly measured using a digital pH meter calibrated against the standardized buffers of pHs 4.0 and 6.8. Free amino acid determination were performed on an automatic analyzer system (A300; MembraPure GmbH, Hennigsdorf, Germany) using the method described by Cui *et al.* (18). Prior to amino acid analysis, samples were mixed with equal volumes of 10% sulfosalicylic acid solution, and subsequently passed through a 0.45-μm pore size membrane filter. Additionally, alcohol determination was consistent with the method adopted by our previously published paper (19).

Urea and cyanide were assayed by spectrophotometrical methods using diacetylmonoxime (20) and isonicotinic acid/barbituric acid (21) as chromogenic agents, respectively. These reactions were detected on a UV-VIS spectrophotometer (LAMBDA25; PerkinElmer).

Determination of EC and potential EC contents The values of potential EC contents were defined as the contents of EC produced after Goji wines were heated at 80°C for 48 h, as determined by Romero *et al.* (22). The proposed procedures aimed to simulate slow EC-forming reactions that occur during a prolonged storage process. The sample cleanup and preconcentration procedures of EC analysis of the fermented wines were based on our previously modified and validated method (23). Briefly, 3 mL of sample solution spiked with *n*-PC as internal standard was added to a centrifuge tube, followed by neutralization to pH 7 with NaOH (1 mol/L). After the vortex, the mixture was transferred into a SPE column and adsorbed for 10 min. The analyte was eluted with 15 mL of dichloromethane. Then the collected elution was dehydrated by anhydrous sodium sulfate and concentrated to 0.5 mL under a gentle nitrogen flow at 35°C. The concentrate was subjected to GC-MS analysis with 1-μL injection volume.

A Trace GC Ultra gas chromatograph-DSQ II mass spectrometer (Thermo Electron Corporation, Waltham, MA, USA) equipped with a HP-INNOWAX capillary column (30.0 m×0.25 mm i.d., 0.25-μm film thickness, Agilent Technologies) was used. The initial temperature for the oven was programmed at 100°C and held for 3 min, and then raised to 130°C at 3°C/min. Then it was heated to 170°C at 8°C/min and increased to 220 at 10°C/min and held for 10 min. A purified helium carrier gas (99.999%) was set at a constant flow of 1 mL/min. Selected ion monitoring mode and electron impact ionization were employed for the MS spectrometer, and the splitless injector port and transfer line were kept at 230 and 250°C, respectively. Major ions *m/z* 62, 74, and 89 were monitored for EC confirmation, and *m/z* 62 was chosen as the quantitative ion in the mode.

Statistical analysis The data reported were calculated from three independent analyses and expressed with average values. One-way analysis of variance, Students'*t*-test, and Spearman's correlation analysis were performed using SPSS software (version 19.0, SPSS Inc., Chicago, IL, USA). Samples with *p*-value less than 0.05 were considered as statistically different.

Results and Discussion

Compositional analysis of Goji fruits To elucidate the physiochemical differences of Goji fruits employed and possible effects of Goji fruit composition on EC formation, major indices to characterize Goji attributes including HGW, water contents, polysaccharide, total sugars, total nitrogen, and ash contents are listed in Table 1. The results showed that there were significant differences in HGW among all Goji samples, while they had similar water contents. Overall, the Goji samples had substantial sugar contents (37.48 to 62.81%) but relatively low nitrogen contents (0.91 to 2.10%), leading to quite a low nitrogen-to-carbon (N/C) ratio. Goji #4 presented the highest N/C ratio, followed by Goji #1, #2, and #3 in sequence. Zhao *et al.* (24) have proven recently that the regulators in nitrogen metabolism are significantly involved in the EC formation in rice wine systems. Meanwhile, different nitrogen sources can result in changes of the expression of the regulators at transcriptional and post-transcriptional levels for yeasts (25). Therefore, the above results indicate that different components from the Goji samples might produce potential effects on the formation of EC in the wines.

Analysis of fermentation process associated with EC formation

In this investigation, a brewing craft similar to the production of grape musts was performed so as to obtain greater pronounced aromatic complexity for Goji wines (26). Figure 1 summarized the changes of major factors involved in EC formation in Goji wines system, including yeast counting, pH, alcohol, urea accumulation, citrulline, and arginine. Figure 1A showed the growth of total yeasts during the fermentation that employed different Goji fruits as raw material. Yeasts sharply increased within 2 days, grew to maximum values at 4 d, and subsequently slightly declined. After fermentation for 12 d, samples #1, #2, and #3 obtained 131.43, 120.00, and 111.43% higher yeast numbers than that of sample #4. This demonstrated that different chemical compositions in Goji fruits had

an effect on the growth of yeasts. As the precursors of EC, such as alcohol and urea, are related to the metabolism of yeasts (24), varied growth rates and physiological conditions of yeasts may in turn affect the formation of EC. Obviously, pH shared a similar changing trend among the four wines fermented by different Goji fruits (Fig. 1B), which decreased gradually with increasing fermentation time and spanned an acidic pH range from 4.2 to 3.8. Arena and Manca (11) reported that specific arginine consumption at pH 3.8 was higher than that at pH 6.5 and diminished with increasing pH. Due to high-level arginine consumption, large amounts of urea are accumulated by the arginine deiminase pathway. Moreover, the chemical reaction associated with the formation of EC requires an acidic pH condition (27). Consequently, the above pH ranges favored to EC increase in the Goji wines. As a major metabolite of Goji wine fermentation, alcohol, which highly influences the formation of EC, is generally present in greater absolute concentrations than other precursors such as urea and citrulline. According to Fig. 1C, it showed that alcohol was produced in the four samples and increased continuously with the fermentation process. Samples #1 and #2 (11.9%, v/v) produced higher alcohol concentration than samples #3 and #4 (11.0%, v/v). Sample #3 had the highest content of total sugar but produced less ethanol content, which was similar to that of sample #4, suggesting that the higher proportion of non-fermentable sugars was contained.

Urea and many other types of nitrogen-containing compounds derived from the raw materials can be consumed as nitrogen resources for yeasts, but the utilization of urea is closely correlated with nitrogen source distribution and contents in the media because of the regulation of the nitrogen catabolite repression (NCR) mechanism (14). According to NCR, urea is a poor nitrogen source, which is usually not degraded by *S. cerevisiae* in the presence of preferred nitrogen such as aspartate, asparagine, and glutamate. As expected, urea contents accumulated rapidly in the initial stage of fermentation but showed significant differences among the Goji wines (Fig. 1D). To explain this difference, Table 2 examined the initial contents of various amino acids derived from Goji fruits. Based on the preference of yeasts to utilize nitrogen sources for growth, preferred nitrogen and poor nitrogen were classified, as proposed by Zhao *et al.* (14). A total of 17 free amino acids were detected and included five preferred amino acids for yeasts. Correlation analysis revealed the positive relationship ($R^2=0.8$) between the contents of accumulated urea and total preferred nitrogen in Goji wines. In particular, the

Table 1. Chemical attributes of different varieties of Goji

Varieties No.	HGW ¹⁾ (g)	Water (%)	Polysaccharide (%)	Total sugars (%)	Total nitrogen (%)	Ash (%)
#1	17.35±0.22c ²⁾	13.32±0.44a	7.16±0.11c	54.84±0.49b	1.40±0.04b	21.59±2.72ab
#2	14.63±0.09b	12.24±0.60a	5.99±0.14b	55.46±0.34b	1.17±0.14a	24.47±1.23c
#3	19.48±0.24d	13.93±0.44a	5.53±0.15b	62.81±0.66c	0.98±0.04a	23.78±0.18bc
#4	8.34±0.08a	16.52±0.24b	3.48±0.02a	37.48±0.51a	2.05±0.12c	18.83±0.51a

¹⁾HGW means hundred-grain weight.

²⁾The different lower-case letters after the data represent significantly statistical difference.

Fig. 1. Changes in yeast cell enumeration (A), pH (B), alcohol (C), urea (D), arginine (E), and citrulline (F) during Goji wine fermentation process. Arginine and citrulline are abbreviated as Arg and Cit, respectively. The bars indicate error ranges, which are expressed by standard deviation ($n=3$).

contents of both arginine and urea were the highest in sample #3. These findings concurred with the previously reported results in the literature (14), stating that the preferred nitrogen sources inhibited urea metabolism at different degrees. More recently, Zhao *et al.* (28) further confirmed that the accumulated urea contents were highly dependent on the usage of arginine by only adding NCR-related amino acids into the media. Therefore, different inhibitory effects on urea utilization induced by the amino acids caused the differences in final urea contents among the Goji wines. However, cyanide was not detected in all Goji wines. This was probably due to the absence of cyanogenic glycosides (a precursor of cyanide) in Goji berries as well as the lack of a high-temperature condition in which urea could be thermally decomposed into cyanide (9).

Citrulline is produced as an intermediate via arginine metabolism, and it can be directly reacted with ethanol to form EC by ethanolysis (29). According to Fig. 1E and 1F, certain contents of these two amino acids from the Goji fruits were observed at the beginning of

fermentation, while they were degraded to undetectable levels after the fermentation was finished. Interestingly, citrulline routinely belongs to a non-preferred nitrogen source and is not consumed at the presence of enough preferred nitrogen sources (14,30). The phenomenon can be explained by the fact that the pattern of nitrogen resource consumption was changed because low temperature during fermentation produced a more relaxed NCR system by decreasing membrane fluidity and permease activity, as reported by Beltran *et al.* (31). On the other hand, the nitrogen-deficient condition in Goji fruit broth further promoted the degradation of the poor amino acids, in accordance with grape musts fermentation (32). This was verified by the very low N/C ratio in the fermentation broth. With respect to EC formation, the transfer of the carbamyl moiety by alcoholysis requires a certain concentration of competing acceptor molecules (29). This revealed that the reaction of citrulline with alcohol is not a major pathway to form EC during the Goji brewing process due to complete consumption of the amino acids.

Table 2. Amino acid profiles of the initial broth from different Goji varieties

Amino acids [Abbr.]	Concentration (mg/L)			
	#1	#2	#3	#4
Preferred nitrogen				
Aspartate [Asp]	94.97±3.97b ¹⁾	68.70±0.98a	106.70±0.91c	99.36±1.12bc
Asparagine [Asn]	338.44±16.70c	189.20±2.39b	199.89±3.61b	152.08±4.82a
Glutamate [Glu]	192.15±5.39b	44.80±0.28a	41.62±0.91a	37.63±1.24a
Arginine [Arg]	80.94±2.56b	77.08±2.60ab	130.13±0.91c	71.85±1.26a
Serine [Ser]	94.84±4.26c	31.47±0.98a	24.66±1.05a	61.25±1.16b
Poor nitrogen				
Threonine [Thr]	26.48±1.85b	19.74±0.69a	20.21±0.50a	32.57±0.98c
Glycine [Gly]	12.33±0.27c	7.11±0.13b	4.39±0.21a	10.43±1.26c
Alanine [Ala]	218.79±8.22d	107.75±3.80b	52.09±0.84a	157.56±4.09c
Citrulline [Cit]	39.09±1.15d	6.45±0.16a	33.01±0.50c	18.72±0.16b
Valine [Val]	30.00±0.44b	26.79±0.30a	25.86±0.91a	26.31±0.55a
Methionine [Met]	37.42±3.97b	23.87±0.98a	30.6±0.49ab	36.84±1.40b
Isoleucine [Ile]	17.29±0.27d	11.15±0.40b	9.59±0.08a	16.18±0.16c
Leucine [Leu]	36.99±0.44b	19.28±0.44a	61.95±0.97c	33.13±1.68b
Tyrosine [Tyr]	65.56±1.15c	42.70±0.83a	40.63±0.77a	58.78±1.54b
Phenylalanine [Phe]	19.31±0.16c	13.64±0.43b	4.75±0.04a	19.35±1.61c
Histidine [His]	304.04±11.05b	204.45±6.10a	302.05±10.40b	304.43±10.20b
Lysine [Lys]	164.83±3.97d	101.12±2.96b	27.74±0.21a	119.65±2.67c

¹⁾The different lower-case letters after the data in the same row represent significantly statistical difference ($p<0.05$).

Analysis of EC contents in Goji wines SPE followed by the GC-MS approach has been proved to possess satisfying accuracy and precision for EC quantification in a complicated sample matrix (7,33). The sensitivity of this method was evaluated by the limits of detection (LOD) and limits of quantification (LOQ). According to the signal-to-noise (S/N) ratio, the LOD and LOQ were calculated as 1.0 and 3.3 µg/L, respectively, which was similar to that of Chinese liquor by the same determination protocol (23). The sensitivity for the EC detection above can meet the requirement of this study.

EC contents were qualified after the fermentation was finished, but the results showed no EC production (Table 3). This is a reasonable phenomenon due to the low temperature and the short period of the fermentation process. Furthermore, the chemical reactions of EC formation have been proved to involve many other contributing factors such as pH and oxidants (11,13). To estimate the final levels of EC produced with a prolonged storage time at ambient temperature, the potential EC contents of fresh Goji wines were measured. The contents of potential EC, remaining urea, and ethyl alcohol in the end products are illustrated in Fig. 2. The four samples exhibited greatly different potential EC contents, with the highest concentration being 96.46 µg/L. Up till now, there is no legislation with respect to EC level limits for Goji wines. Referring to the international allowable contents of grape wines (30 µg/L), potential EC content analysis suggests that Goji wines may encounter the challenges of EC pollution, and effective EC-reducing approaches should be carried out. Goji wines had overall high but similar alcohol concentrations in contrast with urea contents, resulting in little contribution of alcohol to the changes of potential EC contents

across different Goji wines. This finding was confirmed by correlation analysis, which showed that the potential EC contents were positively correlated with urea contents of the wines ($R^2=0.8$).

Table 3 examined the effects of temperature on EC formation in Goji wines. The extreme importance of storage temperature was demonstrated according to the changes of EC contents in the respective samples stored at room temperature and 4°C for 3 months. In particular, compared with the samples stored at 4°C, EC contents were obviously increased for the samples stored at room temperature. This was in agreement with the results of yellow rice wine and Brazilian sugar cane spirits (6,34). In regard to grape wines, Stevens and Ough (10) observed a three-fold EC increment with a 10°C increase of storage temperature. Most studies have focused on the accumulation of EC in the storage process, but EC contents produced in the fermentation process were rarely investigated. Table 3 also compared the EC concentrations of the samples fermented at 12 and 30°C in an attempt to clarify whether the fermentation temperature influences EC formation regardless of its effects on aromatic profiles. It showed that Goji brewing at 30°C produced trace-level EC with values fluctuating between the LOD/LOQ of the quantification method, while no EC was detected for the lower temperature fermentation. The result clearly revealed that the fermentation temperature is also an important factor contributing to EC formation. The higher EC concentrations produced resulted from greater temperature and the higher concentrations of alcohol and urea produced during the fermentation process (data not shown), because the reaction between alcohol and urea belongs to a first-order equilibrium and is temperature dependent (35).

Table 3. Determination of EC contents ($\mu\text{g/L}$) at different temperatures during fermentation and 3-month storage process

Goji wines	Fermentation		Storage	
	12°C	30°C	Room temperature	4°C
#1 ¹⁾	nd ²⁾	0.98±0.00a (<LOD ³⁾)	43.78±1.39c	0.92±0.00
#2	nd	1.93±0.31b ⁴⁾	27.58±0.86b	nd
#3	nd	0.97±0.00a	54.86±3.04d	nd
#4	nd	2.94±0.23c	25.17±0.41a	nd

¹⁾Labels #1, #2, #3, and #4 represent the wines fermented with different varieties of Goji.

²⁾"nd" denotes no EC detection.

³⁾LOD denotes the limits of detection, which is 1.0 $\mu\text{g/L}$ by SPE combined with GC-MS in the study.

⁴⁾The different lower-case letters after the data in the same column represent significantly statistical difference.

Fig. 2. Potential formation contents of EC in different Goji wines. The bars indicate error ranges and are expressed by standard deviation ($n=3$). Different lower-case letters on the columns with the same color represent a significant difference ($p<0.05$) among different samples.

Fig. 3. Validation of alcohol and urea content contribution to EC formation. The final concentrations of 20% (v/v) alcohol and 20mg/L urea were used to induce EC production compared with the control (Sample #2). An asterisk labeled on top of the columns indicates statistical difference obtained from Student's *t*-test ($p<0.05$).

Validation of effects of precursors during storage process To validate EC formation induced by two major precursors in the Goji wine matrix, alcohol and urea were supplemented into the Goji wines. The final amounts of EC produced were evaluated by potential EC contents. Figure 3 showed that the wines containing more precursors produced much higher EC levels compared with the control, indicating the concentration effects of alcohol and urea in the Goji wine matrix. But a close examination of the effects of precursor contents on EC formation revealed that the EC contents of the sample with added urea increased appreciably, while EC just increased slightly ($p<0.05$) after the alcohol concentration was adjusted from 12 to 20% (v/v). This showed that the formation of EC was mainly determined by urea contents in the matrix, which was consistent with the results of Fig. 2. Hence, to control EC generation, it is significantly important to reduce the contents of urea produced during the brewing process.

In conclusion, natural and potential EC contents during the production process of Goji wines were examined, and the effect of different chemical compositions of Goji varieties on EC formation was evaluated. The present results revealed that dominant EC contents were formed in the storage process rather than in the fermentation process; therefore, more attention should be paid to

the storage process conditions in order to reduce EC formation. The chemical compositions of the Goji fruits affected EC precursor accumulation by mediating the yeast metabolism. With regard to detailed mechanisms of urea accumulation, a more elaborated research on the nitrogen regulation pattern for the given yeast is still necessary. In addition, this study can be used as a reference to set maximum allowable EC levels for similar fermented fruit wines, as no regulation for EC in alcoholic beverages has been established in China until now.

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