Determination and formation of Ethyl Carbamate in Chinese spirits

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ABSTRACT

This study aims to develop HPLC-FLD method and verify its applicability for Ethyl Carbamate (EC) determination in Chinese spirits, and to investigate the mechanism for EC generation and its influencing factors. HPLC-FLD with prior derivatization with 9-xanthydrol was used for the EC detection. Optimal reaction condition and the parameters usually tested in the method validation were identified and evaluated. Most appropriate mobile phase is sodium acetate solution with pH value at 7.2. Optimal alcohol strength and reaction time for derivatization are 38—42% and 50 min respectively. Good linearity was obtained with correlation coefficient greater than 0.9998; limit of detection and quantitation were 1.82 and 5.34 μg/L; recovery rate was 96.67% (±6.59%), and method precision was less than 5% (RSD). No significant difference was found in EC determination measured by HPLC-FLD and GC/MS (p > 0.05) indicating HPLC-FLD may be a suitable alternative to GC/MS with greater convenience and lower cost. The average EC content in the Chinese spirit was 39.3 μg/L (ND. to 79.1 μg/L, n = 90), and EC was mainly produced during storage. Raw material profile, brewing time, and storage time and temperature were suggested as main factors influencing EC content; moreover, lowering the storage temperature can effectively reduce EC production. Hydrocyanic acid was concluded as the EC precursor in Chinese spirits. A preliminary multiple regression model based on the storage temperature and time was produced to forecast the EC content in Chinese spirits, and this could be further developed in future research including more influencing factors.

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1. Introduction

The carcinogenic and mutagenic effect of Ethyl Carbamate (EC, urethane, $C_2H_5OCONH_2$) was initially discovered in animals in the 1940 s, and it was classified as a group 2A carcinogen (probably carcinogenic to humans) by the World Health Organization's International Agency for Research on Cancer (IARC) (IARC, 2010). EC is found broadly in fermented food, and has been detected in bread, yoghurt, cheese, soy sauce, vinegar, and alcoholic beverages (Dennis, Howarth, Key, & Mases, 1989; Funch & Lisbjerg, 1988; Hasnip et al., 2007; Kim, Koh, Chung, & Kown, 2000).

In Chinese rice wine and sake, one of the main causes of EC presence is related to the formation of its precursor urea, and urea is produced by yeast from arginine metabolism (Ough, Crowell, & Mooney, 1988; Wang, Yang, Zhai, & Zhou, 2007). Consequently,

the use of urea as a fermentation supplement in winemaking is considered as the factor that most contributes to high values of EC in wines, thus resulting in this practice being banned (Butzke and Bisson, 2002; Bertrand and Triquet-Pissard, 1986; Tegmo-Larsson, Splitter, & Rodriguez, 1989). Another contributing factor to the EC existence is associated with storage conditions such as high temperature and prolonged storage time, which may also exacerbate EC formation in some types of alcohol (Stevens & Ough, 1993; Woo et al., 2001; Wu et al., 2014). In alcoholic spirit drinks, there is usually a small amount of EC produced during the fermentation process, only a low percentage of which will be incorporated into the final distillate (Lurton, Vidal, Estreguil, & Cantagrel, 1992; Riffkin, Wilson, Howie, & Muller, 1989). However, high levels of EC were found in stone fruit spirits as they have a comparatively longer storage time. Moreover, the EC presence in spirit drinks has been suggested to be mainly associated with the release of hydrocyanic acid (Christoph, Schmitt, & Hildenbrand, 1987a; Christoph, Schmitt, & Hildenbrand, 1987b; Mildau, Preuss, Franck,

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& Heering, 1987), and it was produced by decomposing cyanoglycosides from many plants (Lachenmeier, Frank, & Kuballa, 2005).

Chinese spirits, one of the traditional Chinese distilled spirits, has similar ethanol content (normally 35–67% by volume) with other spirits such as vodka, whisky and brandy. Grain is the raw material for the fermentation of Chinese spirits. Sorghum or a mixture of sorghum, wheat, rice, glutinous rice, and corn are most commonly used by spirit manufacturers in China. Chinese spirits can mainly be classified into five categories according to their aroma characteristics: strong flavor type raw material: wheat, rice, corn, sorghum, and glutinous rice), mild flavor type (raw material: barley, pea, and sorghum), Jiang-flavor (soy sauce flavor; raw material: wheat and sorghum), rice flavor type (raw material, rice), and miscellaneous (raw material: sorghum and unhusked rice) (NSPRC, 2009). EC was also found in Chinese spirits (Liu et al., 2011; Tang et al., 2011); however, as a result of the variations in the raw material and brewing methods, the mechanism for the EC production in Chinese spirits still requires further clarification.

EC detection in beverages is not simple due to its low concentration and matrix interference (Herbert, Santos, Bastos, Barros, & Alves, 2002). The technique most widely employed for EC testing is Gas Chromatography with Mass spectrometry (GC/MS), and it involves a complex sample pretreatment process. In order to alleviate the interferences from co-extracted matrix components and improve the method sensitivity, a variety of sample clean-up procedures can be selected including liquid–liquid extraction (LLE), solid phase extraction (SPE), or solid phase micro-extraction (SPME). SPE is the most common method in the sample pretreatment stage of EC extraction in alcoholic beverages, and different types of cartridges can be used in its column such as diatomaceous earth, alumina, and florisil; the analytes are normally eluted by methylene chloride (AOAC, 2000; Hasnip et al., 2007; Kim, Koh, Chung, & Kwon, 2000; Lachenmeier, Nerlich, & Kuballa, 2006; Lim and Lee, 2011; Romero, Reguant, Bordons, & Marqué, 2009; Pan et al., 2013). However, the sample preparation steps for the conventional GC/MS method are time-consuming. Therefore, it is of particular importance to develop a suitable methodology for EC determination in Chinese spirits, which can serve as an alternative to GC-based methods for alcoholic beverage analysis.

Herbert et al. (2002) proposed another technique based on an EC derivatization with 9-xanthydrol in an acidic medium. The structure of the xanthyl-EC enables quantitation by Fluorescence Detector (FLD) after separation by High Performance Liquid Chromatography (HPLC). It was suggested by a number of studies that this newly developed method requires shorter sample preparation time and presents higher sensitivity for EC determination in cider spirits, Korean soy sauce, Chinese rice wine, red wine, and white wine (Fu et al., 2010; Roberto & Bele'n, 2009; Sung, Cheong, & Joo, 2007; Susana, Arminda, Beatriz, & Paulo, 2005). However, as EC analysis in Chinese spirits samples is limited, the aims of this study are to develop an HPLC-FLD based method for EC determination in Chinese spirits with 9-xanthydrol, and further investigates the mechanism for EC generation and its influencing factors.

2. Materials and methods

2.1. Reagents and materials

All solvents used were HPLC quality. Ultrapure water was obtained from a Milli-Q system from Millipore (Milford, MA, USA). Hydrochloric acid 37% (m/m), acetic acid, and sodium acetate were analytical grade (Shanghai, China). Acetonitrile, dichloromethane,

diethyl ether, ethanol and 1-propanol were purchased (CNW, Germany) with chromatographic grade. 9-xanthydrol, EC, and D5-EC were purchased (Sigma, Madrid, Spain) and the purity was above 99.8%. CN^- standard solution (50 mg/L) was purchased from Chinese Institute of Metrology.

Stock solution of 1000 mg/L EC was prepared in ethanol and standard solution was diluted with 40% (v/v) ethanol solution before its application.

2.2. Samples

Ninety samples of Chinese spirits were collected from manufacturing enterprises in Sichuan, Shanxi, Guizhou, Guangxi and Hubei provinces, which included five typical spirits flavor aging from 1 to 5 years. Each flavor had more than three samples for each storage year with alcoholic strength ranging from 35% to 67% (v/v). Samples were all collected by the researchers to ensure the product authenticity.

2.3. Standard and sample derivatization

The derivatization reaction was carried out in chromatographic vials as follows: 0.6 mL of the 0.02 mol/L 9-xanthydrol solution and 0.1 mL of the 1.5 mol/L HCl solution were added to 1 mL of standards or samples. The mixture was shaken for a few seconds to guarantee homogenization. The derivatization reaction was then carried out under room temperature and dark condition. Each sample was filtered through a 0.22 μm membrane before analysis. All samples and standards were prepared in triplicates.

The optimal reaction time for derivatization process and the stability of xanthyl-EC stability were investigated from 20 min to 14 h using 200 μg/L EC standard (obtained from neat standard), a spirit sample with and without addition of 200 μg/L EC standard, respectively (n = 3).

Alcohol strength of a sample was adjusted to 15%, 20%, 30%, 35%, 38%, 40%, 42%, 45%, 50% and 60% (V/V), respectively (n = 3), to investigate the impact of alcohol strength on the response level of HPLC for Chinese spirits.

2.4. HPLC-FLD

Separation and quantification were carried out on a Waters chromatograph 2695 and 2475 fluorescence detector. The column was an Atlantis dC18 column (250 × 4.6 mm, 5 μm). The injection volume was 20 μL. The mobile phase consisted of a mixture of 0.02 mol/L sodium acetate (A) and acetonitrile (B). The flow rate was 0.8 mL/min. The excitation and emission wavelengths were 233 nm and 600 nm respectively for FLD. Quantitation was performed according to an external standard method.

The pH value of mobile phase was adjusted to 6.5, 6.8, 7.0, 7.2, 7.4, 7.6 to analyze its impact on xanthyl-EC chromatogram. Column temperature (30 °C–40 °C) and gradient elution were also optimized by a series of experiments.

2.5. Method validation

A seven-point calibration curve of EC was created for quantification of the following concentrations: 5, 10, 50,100, 200, 300, and 500 μg/L. Standard was diluted with 40% ethanol (v/v). Every level was injected three replicates to acquire the mean value of the peak area. The limit of detection (LOD) and limit of quantification (LOQ) were obtained using free-EC Chinese spirit sample spiked with low concentration EC at the signal-to-noise (S/N) ratio of 3 and 10,