www.analytic-news.com

Publication Date: 15.02.2017

Determination of ethyl carbamate in stone fruit spirits, fruit marc spirits and other spirit drinks – A method validation study

Carmen Breitling-Utzmann, Kerstin Zietemann, Simone Goetz, Manuela Hoeger

Chemisches und Veterinäruntersuchungsamt Stuttgart

Abstract

Ethyl carbamate (EC) is a toxic substance which can occur naturally in significant amounts in stone fruit spirits and other spirit drinks. Commission recommendation (EU) 2016/22 states that a target level of 1 mg L^{-1} EC should be achieved in stone fruit spirits and stone fruit marc spirits. In order to control this target level, a GC-MS method for the determination of EC in spirits was successfully validated in an interlaboratory study to finally become a European standard.

Introduction

Ethyl carbamate (EC) is a compound that occurs naturally in fermented foods and beverages like e. g. yoghurt, wine and beer. The highest levels, however, with amounts up to 18 mg L^{-1}, have been found in stone fruit spirits [1]. Since ethyl carbamate can be formed by reaction of cyanide with ethanol, cyanogenic plants such as stone fruits are prone to develop high levels of ethyl carbamate during processing [2].

EC is classified as toxic by the EU and is regarded as probably carcinogenic to humans (classified in group 2A by the IARC) [3]. Therefore, the Scientific Panel on Contaminants in the Food Chain of the European Food Safety Authority (EFSA) concluded that ethyl carbamate in alcoholic beverages indicates a health concern, particularly with respect to stone fruit brandies. EFSA recommended taking mitigation measures to reduce the levels of ethyl carbamate in these beverages [4].

Commission recommendation (EU) 2016/22 states that "it is recommended to ensure that all the appropriate measures are taken to achieve levels of ethyl carbamate in stone fruit spirits and stone fruit marc spirits as low as possible with the aim to achieve the level of 1 mg/l as a target." [5]

In order to control the EU target level for EC in stone fruit spirits and monitor respective minimization measures, a standardized method for the determination of ethyl carbamate in alcoholic beverages had to be established within the normative system. In the year 2011 the standardization mandate M/463 in the field of methods of analysis for food contaminants was issued by the European Commission. Within the framework of this mandate, a standardized method for the determination of ethyl carbamate had to be developed. For this part of mandate M/463 the CVUA Stuttgart received the tender.

An in-house validated method was already applied for the determination of EC in spirits with a content of total dry extract of less than 10 g L^{-1} within our institution.

For spirits with higher amounts of total extract, several clean-up procedures are described in literature, normally comprising an Extrelut® extraction procedure with dichloromethane, followed by detection with tandem mass spectrometry (GC-MS/MS) [6,7], or gas chromatography-mass spectrometry (GC-MS) [8].

The clean-up step was modified in order to omit the use of dichloromethane. Both methods were then interlaboratory validated by means of a collaborative trial. Method descriptions, design and results of the ring trial are given below.

Experimental

Chemicals and standards

$Ethanol_{abs.}$, ethyl acetate, cyclohexane and n-pentane (each of gradient grade) as well as Extrelut® NT 20 SPE cartridges, refill material and sodium chloride were purchased from Merck (Darmstadt, Germany). The analytical standard ethyl carbamate EC (purity > 99 %) as well as the internal standard ISTD butyl carbamate BC were obtained from Aldrich (Milwaukee, IL, USA), the ISTD d_5-ethyl carbamate d_5-EC (purity > 99 %) was purchased from Dr. Ehrenstorfer (Augsburg, Germany).

For EC, BC, and d_5-EC, three stock solutions of 1 mg mL^{-1} each were prepared in $ethanol_{abs.}$. The EC stock solution was diluted with $ethanol_{abs.}$ to obtain two EC working solutions with concentrations of 100 µg mL^{-1} and 10 µg mL^{-1}, respectively. The ISTD stock solutions (BC and d_5-EC) were diluted to 40 µg mL^{-1} with ethanol 65 %vol to obtain the ISTD working solutions.

Material for the collaborative trial

Unsweetened fruit spirits, sweetened fruit spirits, fruit liqueur as well as 0.5 L glass bottles with an aluminum cap were purchased at a local wholesaler.

Apparatus

Since stone fruit spirits may contain precursors of ethyl carbamate which are transformed into EC under the influence of sunlight, light-protected glass ware (e.g. brown glass) was used during the analysis.

A rotary evaporator equipped with a water bath from Büchi (Flawil, Switzerland) was used for concentrating the eluate after the solid phase extraction (SPE) clean-up. The blender Silverson Model F (Silverson, Waterside, UK) was used for spiking the respective matrices.

Electronic pipettes applicable for volumes of 10–100 µL and 200–1000 µL, respectively, and manual pipettes applicable for volumes of 1–10 mL were obtained from Eppendorf (Hamburg, Germany). Analytical balances capable of weighing down to 0.1 mg or down to 0.01 mg from Mettler-Toledo (Greifensee, Switzerland) were used.

A Thermo GC-MS system (Thermo Fisher Scientific GmbH, Dreieich, Germany) comprised of a Thermo Trace GC and a Thermo Polaris Q Ion Trap, was used for analysis. The system was equipped with a CTC CombiPal autosampler (CTC Analytics AG, Zwingen, Switzerland). Chromatographic separation was achieved using a ZB-WAX column (30 m x 0.25 mm x 0.25 µm) from Phenomenex (Aschaffenburg, Germany).

Ethyl carbamate standard solutions for simplified method, dry extract < 10 g L^{-1}

Note: The injection of samples with a dry extract exceeding 10 g L^{-1} could lead to a rapid loss of sensitivity of the GC-MS system, depending on the type of liner and GC-column used. For samples with higher dry extract content a sample clean-up is recommended.

It is possible to prepare the EC standard solutions to an alcoholic strength with a volume fraction of 40 % or with a volume fraction of 70 %, depending on the GC system chosen to carry out GC/MS analysis. For example, for an ethyl carbamate standard solution with an EC mass concentration of 1.0 mg mL^{-1} and a volume fraction of 70 %, 100 µL of ethyl carbamate working solution (100 µg mL^{-1}) and 400 µL $ethanol_{abs.}$ were pipetted into a 10 mL volumetric flask. The volumetric flask was filled to the mark with an ethanol solution of 65 %vol. For an ethyl carbamate standard solution with a volume fraction of 40 %, an ethanol solution of 35 %vol was used to fill up the volumetric flasks.

After having filled each of the 10 mL volumetric flasks to the mark, 0.25 mL of ISTD butyl carbamate working solution were added. Nine ethyl carbamate standard solutions ranging from 0.0–4.0 mg L^{-1} were prepared in this manner.

It is very important that the volumes of standard solutions and sample solutions are equal (before an optional adjustment of the alcoholic strength). Usually, 10 mL of sample were used.

The solutions were mixed thoroughly and an aliquot transferred into a GC vial.

Ethyl carbamate standard solutions for the method including sample clean up, dry extract > 10 g L^{-1}

Nine ethyl carbamate standard solutions ranging from 0.0–3.0 mg L^{-1} were prepared according to the following scheme.

For example, for an ethyl carbamate standard solution with an EC mass concentration of 1.0 mg L^{-1}, 200 µL of ethyl carbamate working solution (100 µg mL^{-1}) were pipetted into a GC vial, 700 µL of $ethanol_{abs}$ and 100 µL of ISTD d_5-EC working solution were added. After sealing the GC vial with a septum cap the contents were mixed thoroughly.

The mass concentration of ethyl carbamate given in mg L^{-1} is related to a sample volume of 20 mL (see below). If different sample volumes are used, an appropriate dilution factor has to be applied.

Sample preparation

Simplified method (dry extract < 10 g L^{-1}): For samples with a dry extract of less than 10 g L^{-1} no sample clean-up was needed. 10 mL of the sample were pipetted into a brown sample flask. A previously calculated amount of $ethanol_{abs.}$ or water was added to the sample to adjust the alcoholic strength to 70 %vol or 40 %vol, respectively, depending on which volume fraction is preferred for GC/MS analysis. After adjusting the alcoholic strength, 0.25 mL of ISTD BC working solution were added and the sample solution mixed thoroughly. An aliquot of the sample solution was transferred into a brown glass GC vial and submitted to GC-MS analysis.

Method including sample clean-up (dry extract > 10 g L^{-1}): 20 g of Extrelut® material were mixed with 10 g of sodium chloride and transferred to a SPE column. 20 mL of the sample were mixed with 100 µL ISTD d_5-EC working solution in a laboratory beaker and the solution was then transferred onto the Extrelut® material. The beaker was rinsed with 2 mL $ethanol_{abs.}$ After allowing the sample to permeate into the material for 15 min, the extraction material was washed with 2 x 20 mL of n-pentane and the eluate discarded. EC was then eluted with a mixture (1 + 1) of ethyl acetate and cyclohexane in several portions of about 20 mL up to a total of 90 mL of eluant. The eluate was concentrated to approximately 1 mL and then transferred into a GC vial.

GC/MS analysis

The following conditions were used for GC/MS analysis: 1.0 µL of sample solution was injected in splitless mode into a PTV injector which was programmed as follows: 50 °C initial temperature with 1.0 min initial hold, heating ramp to 250 °C with a rate of 14.5 °C s^{-1}, hold time 5 min. Carrier gas flow (helium) was set at 1.2 mL min^{-1} in constant flow, with a splitless time of 1.0 min, and a succeeding split flow of 20 mL min^{-1}. The oven temperature programme started at 50 °C with 1.0 min initial time, followed by two heating ramps (5 °C min^{-1} to 150 °C, then 20 °C min^{-1} to 220 °C), and a final time of 10.5 min, resulting in retention times of about 18 min for EC and d_5-EC, and 20 min for BC, respectively (see Figure 1 and Figure 2). The transfer line temperature was set at 240 °C, the source temperature at 230 °C. The mass selective detector (ionization in EI positive mode, 70 eV) worked in single ion monitoring (SIM) mode recording the ions m/z 44 and 62 for EC and BC, and m/z 64 for d_5-EC with a dwell time of 100 ms for each ion.

Collaborative Trial – Design

Preparation of samples for the method validation study

For the method validation study, four different matrices were prepared by spiking commercial spirits containing no EC with a defined ethyl carbamate solution.

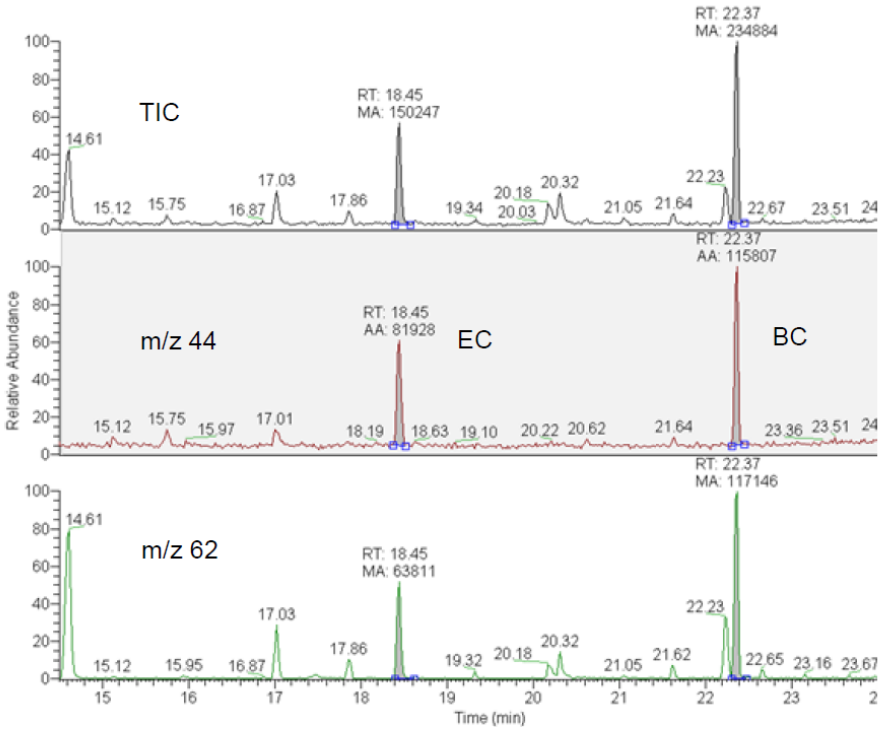

Fig. 1: GC/MS chromatogram (SIM) of a sample solution (stone fruit spirit), containing 0.56 mg L^{-1} EC; internal standard BC.

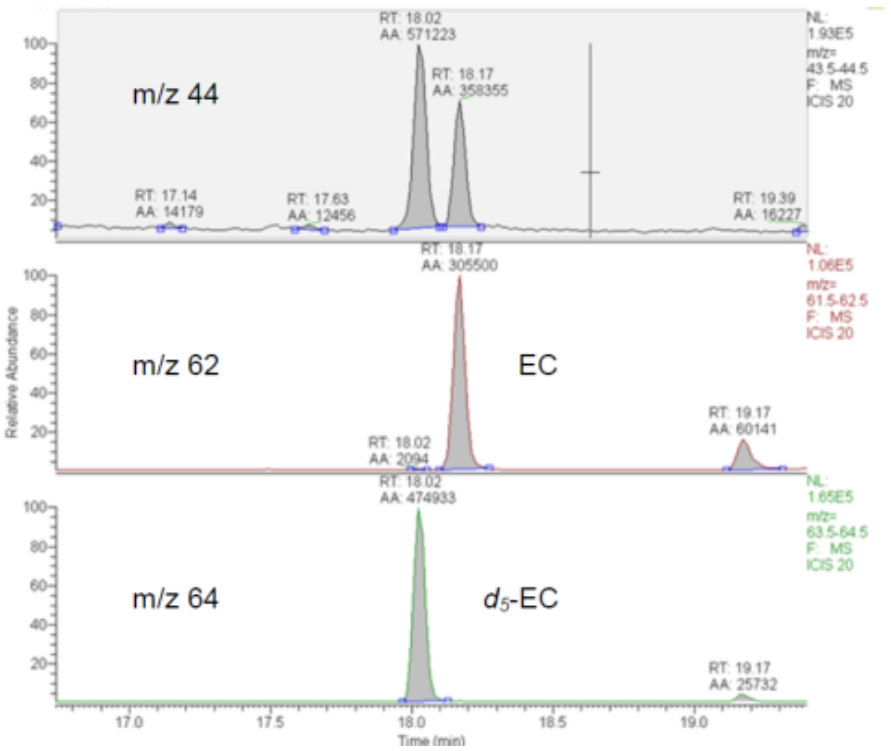

Fig. 2: GC/MS chromatogram (SIM) of a spiked sample solution (liqueur), containing 0.10 mg L^{-1} EC; internal standard d_5-EC.

For the spiking solution, 100.87 mg EC were dissolved in 100 mL $ethanol_{abs}$ to obtain a final concentration of 1.0087 mg mL^{-1}. In a large blender, a defined volume of spiking solution (see Table 1) was pipetted to the respective matrix and mixed for approximately 10 min, achieving the target concentrations given in Table 1. The ring trial material was filled into 0.5 L bottles, which were sealed with aluminum screw caps.

M1 represents a fruit spirit with an EC mass concentration at the EU target level of 1 mg L^{-1}. M2 and M3 are spirits with frequently observed EC levels. M4 simulates a liqueur which was manufactured using in a ratio of 1 + 1 e.g. fruit juice and a stone fruit spirit with an EC mass concentration exceeding the EU target level.

Tab. 1: Preparation of sample material for the method validation study

Sample	Matrix	Fruit brandy / liqueur [L]	Spiking solution [mL]	Target mass concentration [mg L^{-1}]
M1	Low-extract, unsweetened fruit brandy	44	50	1.146
M2		30	11	0.370
M3	sweetened fruit brandy	30	8	0.269
M4	liqueur	30	18	0.605

Tab. 2: Participating laboratories

Country	Official food lab	Commercial lab	Company lab
France	2	-	1
Germany	10	3	-
Italy	-	-	1
Switzerland	2	-	-
UK	-	-	3

Homogeneity and stability tests were performed with the sample materials according to the procedure described in the IUPAC protocol [9]. All test materials proved to be homogeneous and stable.

Participants of the method validation study

A total of 22 laboratories participated in the method validation study, including official food control laboratories, commercial laboratories, and company laboratories (see Table 2).

Method validation study by collaborative trial

Two bottles of each matrix were sent to the participants of the method validation study. All participants were advised to analyze the amount of ethyl carbamate in all four matrices in quadruplicate in order to test repeatability. Matrices M1 and M2 were to be analyzed using the simplified method, for matrices M3 and M4 the SPE clean-up step had to be used.

The results had to be reported within eight weeks, using a previously defined spreadsheet. The chromatographic conditions should also be reported in order to distinguish possible influences caused by the equipment used.

It was advised that apart from the ethyl carbamate content, the alcoholic strength and the dry matter should be determined in each matrix to identify possible deviations of the sample material.

Collaborative Trial – Results and Statistical Evaluation

All 22 participating laboratories reported results. Two laboratories submitted results only for matrices M1 and M2, one laboratory for M1, M2, and M3. One laboratory provided only a duplicate instead of a quadruplicate of results.

The statistical evaluation of the collaborative trial results was performed by QuoData GmbH (Dresden, Germany), according to ASU § 64 LFGB, based on ISO 5725-2 [10]. The following presentation of the collaborative trial results is taken from the evaluation report prepared by QuoData [11].

By using several outlier tests, it was tested whether outliers within the laboratories were present, whether the variances of the laboratories were approximately the same and whether systematic errors affected the means.

A total of six data sets, each with 4 measured values, were identified as outliers and

excluded from the subsequent analysis due to excessive variance of the individual measurement values.

The Shapiro-Wilk test showed that the distribution of the measurement values (outliers excluded) followed a normal distribution.

Table 3 shows the statistical parameters according to ASU § 64 LFGB (based on ISO 5725-2) [10].

The calculated precision data showed good results: the relative reproducibility standard deviations were between 11.4 % for matrix M1 with the highest and 25.1 % for matrix M3 with the lowest concentration of ethyl carbamate [11]. Regarding trueness, no statistical significant deviations from the respective target concentrations of the samples could be detected; recovery was determined to be higher than 90 % and lower than 110 % for all samples. The calculated reproducibility standard deviations meet the criterion of the classical Horwitz standard deviation [12]. None of the computed HorRat values exceeded the limit of 2 which is often considered as critical.

Conclusions

Based on the obtained precision data, both methods (with and without sample clean-up) can be considered as interlaboratory validated for the determination of ethyl carbamate in stone fruit spirits, fruit marc spirits and other spirit drinks. The described method was accepted as a European Standard by CEN/TC 275/WG 13 "Process contaminants" and is designated for publication as EN 16852 [13] in 2017.

References

[1] *Lachenmeier D. W., Schehl B., Kuballa T., Frank W., Senn T., Retrospective trends and current status of ethyl carbamate in German stone-fruit spirits. Food Addit. Contam. 2005, 22, 397*

[2] *Commission Recommendation 2010/133/EU on the prevention and reduction of ethyl carbamate contamination in stone fruit spirits and stone fruit marc spirits and on the monitoring of ethyl carbamate levels in these beverages, OJ L 52/53, 03.03.2010*

[3] *World Health Organization; International Agency for Research on Cancer; IARC Monographs on the Evaluation of Carcinogenic Risks to Humans; Vol.96, Alcohol Consumption and Ethyl Carbamate, Lyon, France 2010, ISBN 978 92 832 1296 6*

[4] *Opinion of the Scientific Panel on Contaminants in the Food chain on a request from the European Commission on ethyl carbamate and hydrocyanic acid in food and beverages, The EFSA Journal 2007, 551, 1 https://www.efsa.europa.eu/en/efsajournal/pub/551*

[5] *Commission Recommendation (EU) 2016/22 on the prevention and reduction of ethyl carbamate contamination in stone fruit spirits and stone fruit marc spirits, repealing Recommendation 2010/133/EU, OJ L6/8, 09.01.2016*

[6] *Lachenmeier D. W., Frank W., Kuballa T., Application of tandem mass spectrometry combined with gas chromatography to the routine analysis of ethyl carbamate in stone fruit spirits.*

Tab. 3: Precision data for the determination of ethyl carbamate in spirits [11]

Sample	Low-extract, unsweetened fruit brandy 1	Low-extract, unsweetened fruit brandy 2	Sweetened fruit brandy	High extract liqueur
Preparation of test material	spiked	spiked	spiked	spiked
Year of interlaboratory test	2012 and 2013	2012 and 2013	2012 and 2013	2012 and 2013
Number of participating laboratories	22	22	22	22
Number of accepted results (laboratories)	22	22	20	19
Number of outliers (laboratories)	3	1	1	1
Number of laboratories retained after eliminating outliers	19	21	19	18
Mean value, $\bar{x}$, mg L^{-1}	1.11	0.400	0.253	0.592
Repeatability standard deviation s_r, mg L^{-1}	0.033	0.022	0.009	0.024
Repeatability relative standard deviation, RSD_r, %	2.99	5.58	3.66	4.07
Repeatability limit r [r = 2,8 x s_r], mg L^{-1}	0.093	0.063	0.026	0.067
Reproducibility standard deviation s_R, mg L^{-1}	0.127	0.074	0.064	0.125
Reproducibility relative standard deviation, RSD_R, %	11.39	18.60	25.06	21.05
Reproducibility limit R [R = 2,8 x s_R], mg L^{-1}	0.354	0.208	0.178	0.349
Recovery, %	97	108	94	98
HorRat value in accordance with [12]	0.72	1.01	1.27	1.22

Rapid Commun. Mass Spectrom. 2005, 19, 108

[7] Hamlet C. G., Jayaratne S. M., Morrison C., Application of positive ion chemical ionization and tandem mass spectrometry combined with gas chromatography to the trace level analysis of ethyl carbamate in bread. Rapid Commun. Mass Spectrom. 2005, 19, 2235

[8] AOAC official method 994.07, Ethyl carbamate in Alcoholic Beverages and Soy Sauce, J. AOAC Int. 1994, 77, 1530

[9] The International Harmonized Protocol for the Proficiency Testing of Analytical Chemistry Laboratories; Pure Appl. Chem. 2006, Vol. 78, No. 1, 145

[10] ISO 5725-2:1994, Accuracy (trueness and precision) of measurement methods and results – Part 2: Basic method for the determination of repeatability and reproducibility of a standard measurement method

[11] Method Validation Study „Determination of ethyl carbamate in spirits“, Evaluation report 2013, QuoData GmbH Quality & Statistics!, Kaitzer Str. 135, 01187 Dresden

[12] W. Horwitz and R. Albert: The Horwitz Ratio (HorRat): A Useful Index of Method Performance with Respect to Precision, J. AOAC Int., 2006, 89, 1095

[13] DIN EN 16852, Foodstuffs – Determination of ethyl carbamate in stone fruit spirits, fruit marc spirits and other spirit drinks – GC-MS method; Lebensmittel – Bestimmung von Ethylcarbamat in Steinobstbränden, Obstbränden und anderen alkoholischen Getränken – GC-MS Verfahren; Deutsche und Englische Fassung EN 16582:2017, to be published