TITLE: VALIDATION OF AN ANALYTICAL METHOD FOR THE DETERMINATION OF ETHYL CARBAMATE IN VINEGARS

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Abstract

A solid phase extraction method (SPE) using Isolute ENV+ cartridges was validated for the determination of ethyl carbamate (EC) in different kinds of vinegars. The method proved to be quite sensitive, precise and accurate, improving the recovery and LQD of other existing methods for the same purpose. For the optimization of the method, different pH values of the sample were tested, resulting 5.5 the most adequate. Among the 14 samples analyzed, only 5 of them had contents of EC above the quantification limits, ranging between 6.73-56.4 µg/L. The highest value was found in red wine vinegar. Taking into account the amount of vinegar consumed in a meal and the limits established for alcoholic beverages in some countries, the levels of ethyl carbamate in the vinegars tested in this work are acceptable.

Keywords: Ethyl carbamate, vinegar, solid phase extraction, gas chromatography-mass spectrometry.

1. Introduction

Ethyl carbamate (EC), or urethane, is genotoxic and carcinogenic in a number of species, including mice, rats, hamsters and monkeys which suggests a potential carcinogenic risk to human [1-4]. This compound is present in many fermented food (yoghurt, cheese or bread) and alcoholic beverages (wine, beer or spirits, particularly in stone-fruit brandies), usually consumed by human population [5]. Ethyl carbamate, potentially toxic, was re-classified in 2007 as probably human carcinogen compound (Group 2A) by the International Agency for Research on Cancer (IARC) [5]. Thus, the presence of ethyl carbamate in beverage and food is a public health concern for government agencies from countries throughout the word [6].

Ethyl carbamate results from the reaction between ethanol and nitrogen-containing compounds (e.g. urea, citrulline, hydrogen cyanide, cyanogenic glycosides, and other N-carbamyl compounds), which has a moderate kinetic formation at room temperature [7]. One of the most common formation pathway of ethyl carbamate production, in acidic medium, is the reaction of urea with ethanol [8,9]. In the case of wine, the yeasts generate urea from the degradation of arginine [10]. Median levels of ethyl carbamate in alcoholic beverages of up to 5 μg/L for beer and wine, 21 μg/L for spirits other than fruit brandy and 260 μg/L for fruit brandy were calculated [11].

There are currently no harmonised maximum levels for ethyl carbamate. In Canada, the first country to introduce maximum levels of ethyl carbamate in a variety of alcoholic beverages, and in the Czech Republic, the limits range from 30 μg/L for wines to 400 μg/L for fruit brandies. The USA has voluntary targets for wines 15-60 μg/L [11]. Recently, the European Union (EU), recommended taking mitigation measures to

reduce the levels of ethyl carbamate in stone fruit spirits and stone fruit marc spirits to get levels of ethyl carbamate as low as possible with the aim to achieve the level of 1 mg/L as a target [12].

Ethyl carbamate has been analyzed employing different analytical instruments. Most of them require pre-treatments of the sample to avoid interferences and increase the sensitivity. Among them, we can mention liquid-liquid extraction, solid phase extraction (SPE) or solid phase microextraction (SPME). Different solvent in liquid-liquid extraction has been employed, dichloromethane [13] or ethyl acetate [14]. Solid phase extraction (SPE) has been widely applied using different types of cartridges such as ENV+ (hyper cross-linked styrene-divinylbenzene copolymer column) [6,15], or diatomaceous earth column [16-20]. Recently, solid phase microextration (SPME) has also been employed in the analysis of wines and spirits [7,21,22].

The most widespread analytical technique used is gas chromatography simple or multidimensional [6,7,13] with different types of detector (FID, MS, MS/MS, etc.). Mass spectrometer detection in selected ion monitoring mode (SIM) increase significantly the ethyl carbamate detection [23].

Ethyl carbamate has also been analyzed by high-performance chromatography with fluorescence detector with a previous derivatization step [24,25]. Moreover, a rapid method as FTIR spectroscopy for stone-fruit spirits analysis [26] and other methods based on more complex techniques such as HPLC-ESI-MS/MS analysis of samples without [27], or with xanthydrol derivatization technique [28] have also been applied.

The presence of ethyl carbamate in vinegars has been scarcely studied [14,17,20]. However, this compound could be present in vinegars since it is a product obtained from a double fermentation, alcoholic and acetous. Ethyl carbamate could come from the raw material (wine) or be formed during process production. Several authors have

reported the formation of urea during the acetous fermentation [29], which could lead to the synthesis of ethyl carbamate that is favoured in acidic medium as vinegar.

The aim of this work was to develop and validate an analytical method for determining ethyl carbamate in different types of vinegars by SPE and gas chromatography-mass spectrometry analysis.

2. Materials and Methods

2.1. Chemicals and standard solutions

Methanol, ethyl acetate and sodium hydroxide were purchased from Merck (Darmstadt, Germany), and MilliQ water. The standards employed were ethyl carbamate (EC) (Aldrich) and propyl carbamate (PC) as internal standard (Dr. Ehrenstorfer GmbH Laboratories, Germany). The stock and working standard solutions of EC and PC for validation studies were prepared in ethyl acetate.

On the other hand, for spiked vinegar samples, the stock and working standard solutions were prepared in methanol, since this solvent allows a better solubilization of EC and PC in vinegar matrix than ethyl acetate.

2.2. Samples

Six wine vinegars were analysed: two white wine vinegars (WWV1, WWV2), a red wine vinegar (RWV), and three Sherry vinegar, one from each category: Sherry vinegar (SHV), “Reserva” (RV) and “Gran Reserva” (GRV), with six months, two years and ten years of ageing in oak wood barrels, respectively. Also, eight fruit vinegars were analysed: two persimmon vinegars (PV1, PV2) and six strawberry vinegars (SV1, SVF2, SV3, SV4, SV5, SV6). For validation studies, one white wine vinegar was employed. Wine vinegars were acquired in the market and fruit vinegars were produced in the lab.

2.3. Solid phase extraction

The SPE method employed was a modification of the one used by Jagerdeo et al. [6]. We used cartridges of 6 mL containing 500 mg of ISOLUTE ENV+ (Biotage, Uppsala, Sweden) as extraction phase. The extraction was carried out in a Visipred vacuum manifold (Supelco, Bellefonte, PA). The cartridge was conditioned with 2 ml of methanol followed by 3 ml of MilliQ water. Then, 25 ml of vinegar were passed through the cartridge at a flow rate of 3 ml/min. Samples were previously adjusted to a pH 5.5 with NaOH and spiked with 100 µL of propyl carbamate (6 mg/L). The sorbent was dried by letting air pass through it at -0.6 Bar. EC and PC were eluted from cartridge with 3 ml of ethyl acetate. The organic phase of the eluate was carefully collected with a pipette and afterwards concentred under vacuum to a final volume of 2 ml. 300 µL of the extract were placed into a vial fitted with an insert that was tightly capped for the injection in the gas chromatograph. This extraction procedure was carried out in duplicate for each sample.

2.4. Quantitative analysis

For the quantification in validation studies, we made calibration curves of both standards employing ethyl acetate solutions and injecting them, in triplicate, directly in the gas chromatograph. Concentration ranges were 3-520 µg/L for EC (five different levels of concentration) and 2.88-1000 µg/L for PC (six different levels of concentration). The calibration curves were built representing the areas of the target ion (m/z=62, in both cases) againts the concentrations of analyte.

For the samples quantification, a calibration curve was done using one spiked vinegar with EC at five different levels of concentration (3.7-334 µg/L) which was extracted with the same method employed for the samples. Now, the calibration curve was made using the relative area of EC (ratio between the peak area of target ion of EC and the peak area of internal standard) and the concentration of analyte added to the sample.

2.5. Chromatographic conditions

Extracts were analysed in a gas chromatograph Agilent 6890 GC system coupled to an Agilent 5975 inert quadrupole mass spectrometer. For the separation of the compounds we employed a CPWax-57CB (Varian) capilar column of 50 m × 0.25 mm and 0.20 µm film thickness (Varian, Middelburg, The Netherlands). 4 µL of the extract were injected in the splitless mode with a purge flow of 70 mL/min and purge time of 1 minute. The injector temperature was 220ºC. The carrier gas was He at a constant flow rate of 1mL/min. Oven temperature program was as follows: the initial temperature 40ºC and then was increased 2.5ºC/min until 150ºC for 2 minutes and afterwards increased 15 ºC/min until 220ºC. The quadrupole, source and transfer line temperatures were maintained at 150, 230 and 280 °C, respectively. Detection was carried out in the SIM mode, the monitored ions were: 44, 62 y 74. Extracts were injected in duplicate and the identification was done comparing the peak retention times with their respective standards.

2.6. Validation parameters

For method validation the following parameters were evaluated: linearity, sensitivity (LOQ), precision (repeatability and intermediate precision) and accuracy (recovery studies). For the recovery studies, a white wine vinegar was spiked with five different concentration levels of EC in the range of 3.7 to 161µg/L.

The linearity of the method was determined by two ways: considering the correlation coefficient obtained from the regression line made with spiked vinegar at five different levels of concentration (described in 2.4 section); and plotting the response factor (relative area of peaks divided by their respective analyte concentrations) as a function of analyte concentrations [30].

The quantification limit (LOQ) was calculated as the concentration of ethyl carbamate

in the sample that produces a signal ten times higher than the average of relative area of background noise of the chromatogram baseline.

To study the repeatability of the method, 5 successive extractions of a vinegar sample spiked with 60 µg/L of ethyl carbamate were performed. On the other hand, intermediate precision was evaluated using the same sample referred before and performing the extraction on 5 different days by two different analysts over a month of work.

3. RESULTS AND DICUSSION

3.1. Sample pre-treatments

Some authors which have determined EC in vinegars made a previous neutralization of the samples because this improves the shape of EC peak [14,17,20]. Taking into account this fact, we tested the effect of different pHs in the recovery of EC and PC in vinegar samples spiked with the standards. The pH range assayed was from 2.5, pH of vinegars, to neutrality (pH=7). The pH value of samples was modified with the addition of NaOH. These trials showed that peak areas obtained with vinegar without NaOH addition, were aproximately the half that neutralized vinegar (pH=7) (Figure 1). However, the peaks in the last case had a pronounced tail. At pH 5.5, the side of peaks area was similar to the neutralized winegar but the shape of peaks was much better than in the neutralized samples (Figure 2).

3.2. Method validation

The method was evaluated with respect to linearity, sensitivity (LOQ), precision (repeatability and intermediate precision) and accuracy (recovery studies).

One of the most important issues in a extraction process is the ability to recover the highest amount of the analyte of interest. Thus, the first aspect assessed was the recovery. The average recovery rate (Table 1), in the accuracy assays, was 94.1%,

176 which is a very suitable result according to those proposed by AOAC [16]. Our
177 recovery percentage was higher than those achieved by other methods for EC
178 determination in vinegars (below 83%) [17,20].
179 The good linearity of the method in the used range of concentration was verifyed by a
180 0.9998 correlation coefficient of the regression line between the relative area of EC and
181 the concentration of analyte added to the sample. On the other hand, the line obtained
182 after plotting the response factor as a function of analyte concentrations was horizontal
183 over the concentration range. Two parallel lines are drawn in the graph at 0.95 and 1.05
184 times the average values of the response factors and there were no intersections of the
185 points of response factor with these parallel lines. Both results confirmed the linearity of
186 the method.
187 The LOQ was defined as the lowest concentration of EC in a sample that can be
188 determined quantitatively with acceptable precision and accuracy under the stablished
189 conditions of the method. This value was 1.26 µg/L. If we compare with the LOQs
190 obtained by other authors that ranged between 9.16µg/L-110 µg/L [6,7,20,21,31,32],
191 our method proved to be sensitive enough, improving the values of LOQ achieved up to
192 the present.
193 The precision of the method was evaluated by repeatability and intermediate precision
194 assays. We checked the repeatability of the method by the relative standard deviation
195 (RSD) obtained after repeating the extraction assay of spiked vinegar 5 times
196 successively, resulting a 2.5% (Table 2). In the intermediate precision evaluation, the
197 RSD obtained was 6.5% (Table 2). Both values are in agreement with the values
198 proposed by AOAC [16], showing that the method is quite precise.
199 **3.3. Samples analysis**
200 Once we set up the method, the procedure was applied to different types of vinegars.

Data are presented in Table 3. Among the 14 samples, only 5 of them presented levels above the quantification limits, ranging between 6.73-56.4 µg/L. The highest value was found in red wine vinegar. As mentioned in the introduction, only some countries have established their own maximum limits for the EC content in alcoholic beverages [11], but there are not legal limits for vinegar. Except in the case of red wine vinegar, the EC content in the samples is below those values. Other authors had already described the presence of EC in Sherry vinegar [17], founding concentrations of 33 µg/L. The Sherry vinegars analysed in this study had a lower amount of EC than in the above mentioned work. These levels are far away compared to those found by other researchers in vinegars from Taiwan (107.5-250.5 µg/L) [33].

4. Conclusions

Due to the natural acidity of vinegar, a modification of pH at 5.5 previous to the SPE was necessary in order to get an adequate recovery rate and peak resolution. The present method is quite sensitive, precise and accurate, improving the recovery and LQD of other existing methods for the same purpose. Considering the amount of vinegar consumed in a meal and the limits established for alcoholic beverage in some countries, we could conclude that the levels of ethyl carbamate in the vinegars tested in this work were acceptable.

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Figure Captions

Figure 1. Overlay of chromatograms from spiked vinegars A and B. Vinegar A: with neutralization (continuous line); vinegar B: without neutralization (dashed line).

Figure 2. Overlay of chromatograms from spiked vinegars A and C. Vinegar A: with neutralization (dashed line); vinegar C: pH 5.5 (continuous line).

Figure 1

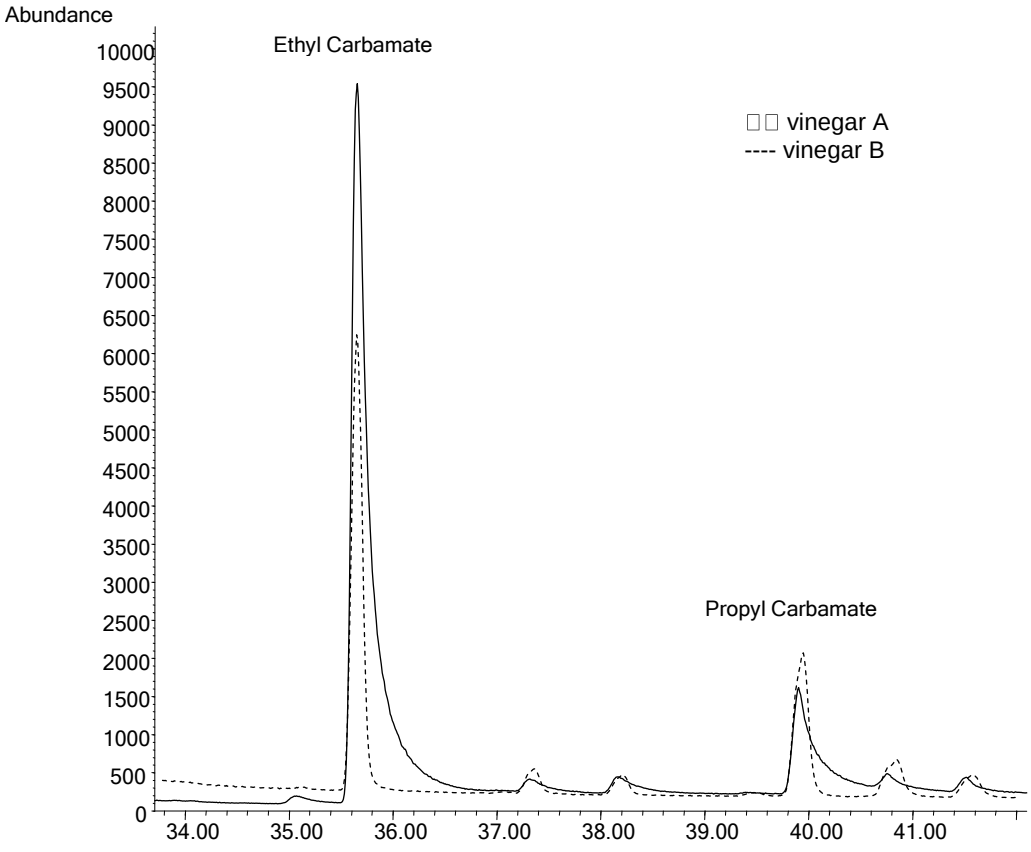
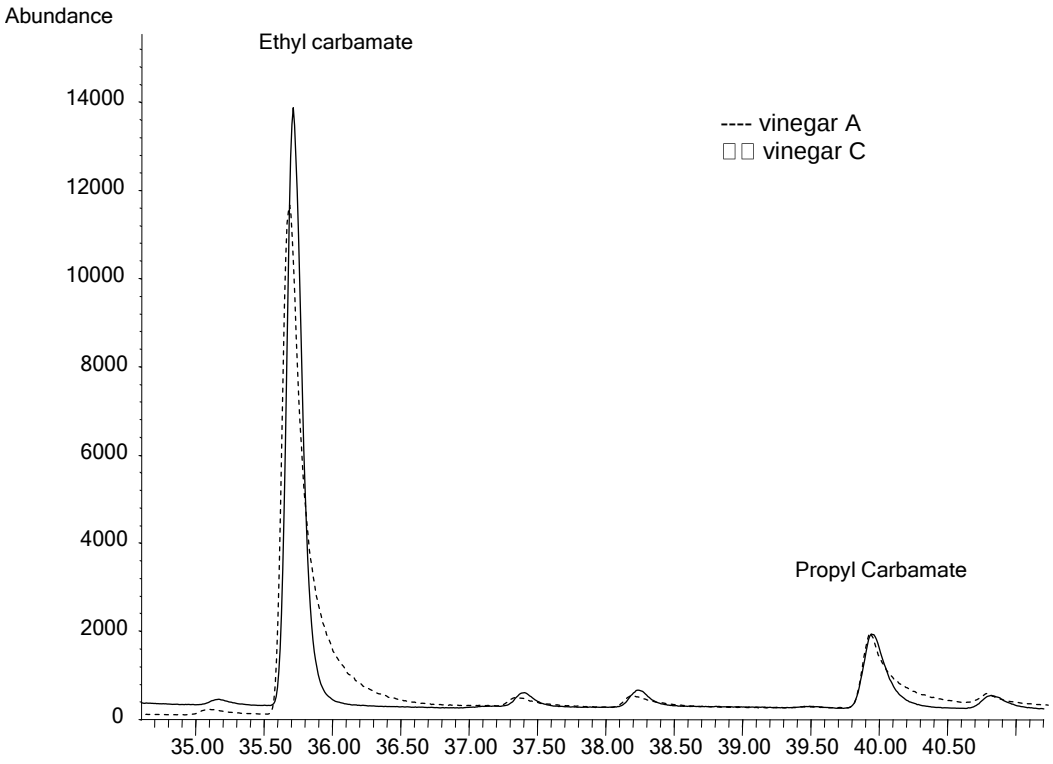

Abundance
Ethyl Carbamate
vinegar A
vinegar B
Propyl Carbamate
10000
9500
9000
8500
8000
7500
7000
6500
6000
5500
5000
4500
4000
3500
3000
2500
2000
1500
1000
500
0
34.00
35.00
36.00
37.00
38.00
39.00
40.00
41.00

Figure 2


Abundance
Ethyl carbamate
14000
12000
10000
8000
6000
4000
2000
0
---- vinegar A
vinegar C
Propyl Carbamate
35.00 35.50 36.00 36.50 37.00 37.50 38.00 38.50 39.00 39.50 40.00 40.50

330 Table 1. Values of accuracy assay.

331

Accuracy assay	EC Added (µg/L)	Recovery (%)	Mean Recovery (%)
Experimental data	3.7	99.0	94.1 ± 3.1
	35	90.5	
	77	92.6	
	115	94.1	
	161	94.3	
AOAC range of suitable values [16]	10-100	-	60-115

332

333 Table 2. Values of precision assay.

334

Precision assay	EC Added (µg/L)	Repeatability (%RSD)	Intermediate Precision (%RSD)
Experimental data	60	2.5	6.5
AOAC maximum suitable values [16]	10-100	5.3-7.3	5.3-7.3

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339 Table 3. Ethyl carbamate concentrations in vinegar samples (µg/L).

340

Sample	Ethyl Carbamate (µg/L)
WWV1	nq
WWV2	6.46 ± 0.01
RWV	56 ± 3
PV1	nd
PV2	nd
SV1	nq
SV2	nd
SV3	nq
SV4	nq
SV5	nq
SV6	nq
SHV	6.7 ± 0.9
RV	14 ± 2
GRV	1.68 ± 0.08

nd: peak not detected.

nq: concentration under quantification limit.

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