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Analytical Methods

Improved sample preparation for GC–MS–SIM analysis of ethyl carbamate in wine

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ABSTRACT

An improved sample preparation procedure for analysis of carcinogenic ethyl carbamate (EC) in wine by GC–MS–SIM is proposed. Differences over AOAC reference procedure were: (1) use of EC-d_5 as internal standard instead of less similar propyl carbamate; (2) extraction by diethyl ether instead of more toxic dichloromethane, and (3) concentration by vacuum automated parallel evaporation instead of more time and work consuming rotary evaporation. Mean recovery was 104.4%, intraday precision was 6.7% (3.4 μg L^{-1}) and 1.7% (88.5 μg L^{-1}), regression coefficient was 0.999 in the linear working range of 3–89 μg L^{-1}, and limits of detection and quantification were 0.4 and 1.2 μg L^{-1}. Applicability was demonstrated by analysis (in triplicate) of 5 wine samples. EC concentration ranged from 5.2 ± 0.2 to 29.4 ± 1.5 μg L^{-1}. The analytical method is selective, accurate, repeatable, linear, and has similar method performance as the reference method along with the several mentioned advantages.

1. Introduction

Ethyl carbamate (EC, $C_2H_5OCONH_2$), a multi-site carcinogen in experimental animals and probably carcinogenic to humans (IARC group 2A), occurs in many fermented foods, in particular alcoholic beverages, where it is thought to be formed from the reaction between ethanol and nitrogen-containing compounds (EFSA, 2007; Lachenmeier et al., 2010). With respect to wine, urea and citrulline – derived mainly from the yeast and lactic acid bacteria metabolisms of arginine – are considered important nitrogen-containing precursors; the rate of EC formation in wine increases with temperature and storage time (Butzke & Bisson, 1997; Monteiro, Trousdale, & Bisson, 1989; Uthurry, Suarez Lepe, Lombardero, & Garcia del Hierro, 2006).

According to data of the European Food Safety Authority, a median of 5 μg L^{-1} and a P95 (95th percentile of values) equal to 78 μg L^{-1} were found in 23,278 wine samples from EU Member States. There are currently no harmonized maximum EC levels for table wine in the EU, but Canada and USA recommend maximum values of 30 μg L^{-1} and 15 μg L^{-1}, respectively (EFSA, 2007).

The standard method for EC determination in wine is the AOAC method 994.07 (Canas, Burns, Joe, & Diachenko, 1994), also adopted by OIV (method MA-AS315-04; OIV, 2013a), and as reference method in the European Union (Commission Regulation, 1999). The AOAC method involves analysis by GC–MS–SIM (gas chromatography coupled to mass spectrometry in selected ion monitoring) after the following sample preparation procedures: (1) addition of propyl carbamate as internal standard; (2) cleanup through diatomaceous earth columns; (3) EC extraction by dichloromethane, and (4) eluate concentration using vacuum rotary evaporation. This technique has been used by several authors (Masqué et al., 2011; Romero, Reguant, Bordons, & Masqué, 2009; Uthurry et al., 2004, 2006) for EC analysis in table wine in recent years. Limiting steps in the standard sample preparation procedures are: (1) use of considerable amounts of a chlorinated toxic solvent (dichloromethane) for extraction; (2) use of intensive labor effort and prolonged time during the concentration step, and (3) use of an internal standard with a lower degree of similarity to control extraction and chromatographic responses. To overcome the solvent limitation, some alternative wine preparation procedures have been proposed, such as the use of solid-phase microextraction (SPME) with a carbowax/divinylbenzene (CW/DVB) fiber (Whiton & Zoecklein, 2002) or solid-phase extraction (SPE) with minimal use of solvents (Jagerdeo, Dugar, Foster, & Schenck,

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2002). Although these alternative preparations have advantages over the standard procedure, they have not been extensively adopted for EC analysis in wine and are not without problems. For instance, the CW/DVB fiber is no longer commercially available (Liu, Xu, & Zhao, 2012). Furthermore, the alcohol part in the sample may influence the SPME extraction yield (Lachenmeier, Nerlich, & Kuballa, 2006) and the method proposed by Jagerdeo et al. (2002) involves a previous time-consuming step for ethanol removal from wine by vacuum.

From a conventional perspective of analysis (AOAC method 994.07), this paper introduces and validates the time and work efficient use of a vacuum automated parallel evaporator for EC analysis in table wine, which allows for the simultaneous evaporation of various wine eluates to a specified volume. Other changes in the AOAC method were carried out, such as the use of the more similar deuterated ethyl carbamate (EC-d_5) as internal standard (which was not commercially available at the time when the AOAC procedure was developed) and the less toxic diethyl ether (instead of dichloromethane) as extraction solvent, which have been suggested in some previous studies (Fauhl & Wittkowski, 1992; Huang et al., 2013).

2. Materials and methods

2.1. Wine samples

Five different bottled, recorded, commercial table wines (W01–W05) were collected in triplicate (same batch code) at Brazilian wineries in May 2012. Three wines (W01, W02, and W03) were representative of two large wineries located in the wine producing region of São Francisco Valley, Northeast Brazil; two wines (W04 and W05) were representative of one large winery located in the wine producing region of Campanha Gaúcha, South Brazil. According to label information, the wine varieties/vintages were the following: Chenin blanc/2010 (W01), Syrah/2010 (W02), Syrah/2008 (W03), Merlot/2010 (W04), and Merlot/2010 reserve (W05). Once collected, the bottles were stored horizontally in a wine cellar at 18 ± 1 °C until analysis.

2.2. Physicochemical characterization of wine samples

The following parameters (principles of methods are given in brackets) were determined (in duplicate analysis) as described by OIV (2013b): total acidity (potentiometric titration using sodium hydroxide), volatile acidity (steam distillation and titration with sodium hydroxide), pH (potentiometry), alcoholic strength by volume at 20 °C (steam distillation followed by measurement using a hydrostatic balance), density (densimetry using a hydrostatic balance), total dry extract (calculated indirectly from the specific gravity of the alcohol-free wine), free sulfur dioxide (direct titration with iodine), total sulphur dioxide (free sulphur dioxide + iodometric titration after alkaline hydrolysis), and reducing sugars (wine clarification by lead acetate; determination by iodometric titration after reducing action on an alkaline copper salt solution). Polyphenols, as total polyphenol index (TPI), were estimated by spectrophotometry at 280 nm (Harbertson & Spayd, 2006). Equipments used in the analyses included: oenochemical electronic distilling unit, model Super DEE, attached to steam distillation unit, model VADE 3 (Gibertini Elettronica SRL, Milano, Italy); hydrostatic balance, model Super Alcomat (Gibertini Elettronica SRL, Milano, Italy); and a spectrophotometer SP 220 (Biospectro Ltda, Curitiba, Brazil).

2.3. Ethyl carbamate analysis in wine

2.3.1. Chemicals and materials

Extrelut NT 20 columns (they contain a mixture of diatom resin and NaCl), Uvasol *n*-pentane (for spectrometry), and ethanol (absolute, pro analysi) were purchased from Merck (Darmstadt, Germany). Diethyl ether (for spectrometry) from Vetec Química/Sigma–Aldrich (Duque de Caxias, Brazil), ethyl carbamate (98.5%) from Dr Ehrenstorfer (Augsburg, Germany), and ethyl-d_5 carbamate (99%; isotopic purity 98 atom % D) from Sigma–Aldrich (St. Louis, USA). Ultra-pure water (Milli-Q system) was used throughout to prepare solutions.

2.3.2. Standards and solutions

For ethyl carbamate (EC) stock solution, 2.8 mg were placed into a 500 mL volumetric flask, diluted to volume in ethanol (abs.) and stored at −20 °C protected from light. For deuterated ethyl carbamate (EC-d_5, internal standard) stock solution, 5.1 mg were place into a 250 mL volumetric flask, diluted to volume in ethanol (abs.) and stored at −20 °C protected from light. For calibration solutions, 15 μL, 35 μL, 75 μL, 155 μL and 395 μL of the EC stock solution were given into five 25 mL volumetric flasks which were then filled to volume using a freshly prepared 13% vol. ethanol solution (simulating a table wine matrix) and stored at 3 ± 1 °C. For matrix effect assessment, the ethanol solution was replaced by wine sample W04 (the lowest in terms of EC concentration found in a previous round of analyses). Final EC concentrations in each calibration standards were 3.4 μg L^{-1}, 7.8 μg L^{-1}, 16.8 μg L^{-1}, 34.7 μg L^{-1} and 88.5 μg L^{-1} which cover the concentration range most likely to be found in table wines (EFSA, 2007). All calibration solutions were treated similar to wine samples prior to measurement (i.e. calibration solutions were extracted and concentrated in the same way as the wine samples).

2.3.3. Extraction

Extraction and concentration procedures were adapted from Lachenmeier et al. (2009). Each calibration solution or wine sample (25 mL) were spiked with 40 μL of internal standard stock solution (final concentration of EC-d_5 equal to 32.6 μg L^{-1}) and directly applied to the Extrelut column. After 15 min of equilibration, the column was washed with 2 × 20 mL of *n*-pentane (aiming at reducing non-polar interferences of the wine matrix). The washing was discarded. Next, the analytes were extracted using 4 × 30 mL diethyl ether and the eluate collected in a 250 mL glass bottle (Schott Duran, Germany) and closed with screw PTFE-lined cap. The bottle was then left at −20 °C for 48 h for the removal of residual moisture. It may be of interest to note that the elution flows of *n*-pentane and diethyl ether were increased considerably by manually applying an over-pressure on top of column with a small hand rubber bellow.

2.3.4. Eluate concentration using a vacuum automated parallel evaporator

Parallel evaporation was performed using a Syncore Analyst with a 6 position rack attached to a vacuum pump/controller V-700/V-855, and recirculating chiller F-108 (Büchi Labortechnik AG, Flawil/Switzerland). Six sample glass vessels with working volumes of 25–250 mL were used. The Syncore Analyst was equipped with a flushback module that condensed the vapor at the top of the vessels, gently rinsing the glass walls. The sample vessels had 3 mL appendices at the bottom, cooled during evaporation, to facilitate the collection of the defined volume and to avoid evaporation to dryness (Fig. 1).

The ethereal eluates at −20 °C were filled into the Analyst sample vessels, leaving behind (attached to the glass bottles) residual moisture as ice. The heating temperature was set at 40 °C and