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Analysis of Ethyl Carbamate in Wines Using Solid-Phase Extraction and Multidimensional Gas Chromatography/Mass Spectrometry

ESHWAR JAGERDEO,*,† SUMER DUGAR,† GREGORY D. FOSTER,‡ AND HILARY SCHENCK§

Bureau of Alcohol, Tobacco and Firearms, 1401 Research Boulevard, Rockville, Maryland 20850, Department of Chemistry, George Mason University, Fairfax, Virginia 22030-4444, and Gettysburg College, Gettysburg, Pennsylvania 17325

The method describes a rapid and accurate procedure for the analysis of ethyl carbamate in wines. The separation of the ethyl carbamate (EC), the target analyte, from alcohol and the sample matrix is a challenge to many analytical chemists. After alcohol removal from the sample, EC was extracted and concentrated by solid-phase extraction. For analysis of EC, large-volume injection on a programmable temperature vaporization (PTV) inlet was used followed by multidimensional gas chromatography/mass spectrometry (MDGC/MS) using electron-impact ionization (EI). For quantitation, the ratio of ions produced during EI at *m*/*z* 62 (EC) and 64 (isotopically labeled EC) was monitored. The use of solid-phase extraction and MDGC/MS removes the majority of the matrix interference encountered in other methods. A linear dynamic range was established from 0.387 to 1160 ng/mL, with a limit of detection at 0.1 ng/mL and limit of quantitation at 1 ng/mL.

KEYWORDS: Ethyl carbamate; multidimensional gas chromatography/mass spectrometry; heart-cutting; wines; carcinogen

INTRODUCTION

Ethyl carbamate (EC), also known as urethane, is a known carcinogen that is found in various alcoholic beverages and food (*1*). The presence of EC in beverages and food is a public health concern for the Food and Drug Administration and government agencies from countries throughout the world.

EC was found to cause benign and malignant tumors in various species of experimental animals (*2*). This was first demonstrated in 1943 in an investigation of EC and factors influencing the formation of lung tumors in mice. The presence of EC in alcoholic beverages first received attention in 1971 when it was reported that diethyl pyrocarbonate (DEPC) reacts with ammonia at neutral or alkaline pH to produce EC (*3*−*6*). During this period, DEPC was widely used as an antimicrobial food additive for beverages. On the basis of this finding, DEPC use as a food additive was prohibited. It was later demonstrated that urea, a natural byproduct of fermentation, is the main precursor of EC in alcoholic products (*1*). Urea was found to be a product of the yeast metabolism of the amino acid arginine (*7*). Any urea that was not used as a yeast nutrient during the fermentation could eventually react with ethanol to produce EC. Thus, in fermented alcohol beverages, EC is formed as a natural byproduct. Urea, once a widely used nutrient supplement in wine fermentation to avoid stuck fermentation, is no longer recommended for use in winemaking (*8*, *9*). Concern in the United States over EC in alcoholic beverages and wines began in November 1985 when it was reported that Canadian authorities had detected this chemical in certain wines and distilled spirits. The average levels of EC for various product classes varied dramatically, with distilled spirits being the highest, followed by wine (*1*). In 1987, the U.S. Food and Drug Administration (FDA) formally accepted a proposal by the Distilled Spirits Council of the United States (DISCUS) to work on reduction of EC levels in distilled spirits (*10*). In 1988, the FDA formally accepted a voluntary program proposal submitted by the Wine Institute and American Association of Vintners, representing the U.S. wine industry, in working to reduce EC. There is a continued effort by ATF to develop methods that would determine and accurately quantitate EC in targeted samples.

There are numerous reported methods for the analysis of EC using liquid/liquid extraction procedure and extraction columns made of diatomaceous earth material (*11*−*13*). These methods are very labor-intensive and use large volumes of methylene chloride as the extraction solvent (*14*, *15*). In addition, there are matrix interferences in the determination of EC at low levels in wines. The new proposed method describes the use of a solid-phase extraction procedure that eliminates the use of methylene chloride. In addition, this method is coupled with a MDGC/MS for the separation and identification of EC.

* To whom correspondence should be addressed. Phone: 301-217-5728. Fax: 301-413-9463. Email: ejagerdeo@atfhq.atf.treas.gov.
† Bureau of Alcohol, Tobacco and Firearms.
‡ George Mason University.
§ Gettysburg College.

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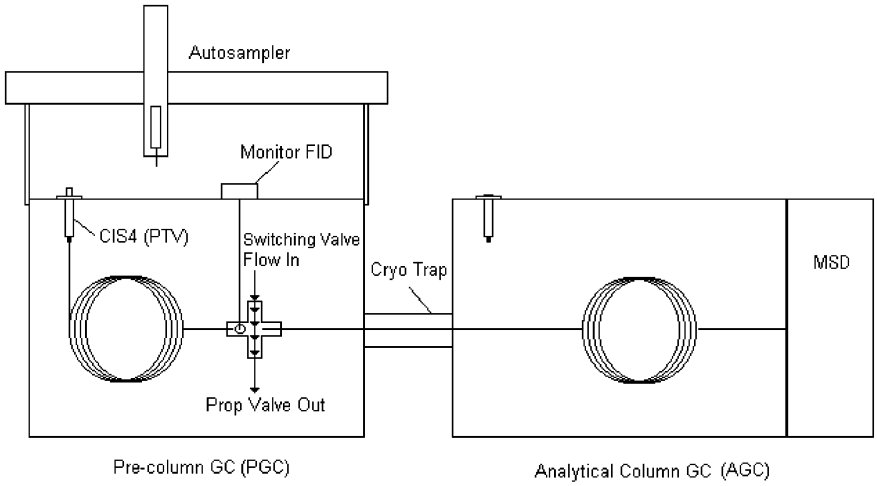

Figure 1. Schematic diagram of the multidimensional GC/MS system (courtesy of Gerstel Inc.).

MATERIALS AND METHODS

Materials. Methanol and ethyl acetate (HPLC grade solvents) were purchased from Burdick & Jackson or equivalent. All gases (helium, hydrogen, air, and liquid nitrogen) were purchased from Air Products and Chemicals Inc. (Hyattsville, MD). Solid-phase cartridges containing 500 mg of ENV+ (a hyper cross-linked styrene−divinylbenzene copolymer) in 25-mL tubes were used for the extraction of the wine (Jones Chromatography USA, Lakewood, CO). Anhydrous sodium sulfate cartridge was used to remove residual water from the final elution (Jones Chromatography USA, Lakewood, CO). A 12-port SPE vacuum manifold (Visiprep) was used for the solid-phase extraction (Supelco Inc., Milwaukee, WI). EC ($C_3H_7O_2N$) was purchased from Supelco Inc., Milwaukee, WI (purity 99%). Labeled EC ($C_2{}^{13}CH_7O_2{}^{15}N$) was purchased from C/D/N Isotopes Inc., Pointe-Claire, Quebec, Canada (purity 99%), and Cambridge Isotopes, Andover, MA (purity 99%).

Standards. For the EC stock solution, 97.8 mg was placed into a-200 mL volumetric flask and diluted in methanol. A working solution was prepared by transferring 2 mL of EC stock solution into a 50-mL flask and diluting to volume with water.

For the labeled EC stock solution, 48.9 mg was placed into a 100-mL volumetric flask and diluted with methanol. A working solution of labeled EC solution was prepared by transferring 2 mL of stock solution into a 50-mL flask and diluting to volume with water. Using the working solutions of EC and labeled EC, standard curves (eight points) were prepared in wine to produce final concentrations ranging from 0.387 ppb to 1161.6 ppb. Quality control, precision, and accuracy assessment, intraday, and interday samples were prepared in a similar manner. For all extracted samples, 25 mL of wine was used, and it was spiked with 0.3 mL working solution of labeled EC internal standard before it was placed in the centrifuge vacuum concentrator.

Sample Preparation. Prepared standards and samples were placed in a centrifuge vacuum concentrator (Speed Vac (Savant Instrument Inc., MA)). The length of time the samples need to be in the concentrator depends on the initial concentration of ethanol in the sample. For this method, samples were placed in the vacuum system at the end of the day and removed the following morning for extraction (∼14−16 h). After the removal of the ethanol, all samples were brought back to initial volume (25 mL) with water. The solid-phase cartridges were placed on the vacuum manifold and conditioned with 2 mL of methanol followed by 3 mL of water prior to extraction. The samples were applied to the cartridge and allowed to flow through the cartridge under gravity only. After the sample had passed through the cartridge, it was washed with 2 mL of water under gravity. The cartridges were aspirated under vacuum (∼10 psi) until most of the water was removed. Cartridges containing 2.5 g of Na_2SO_4 were stacked underneath the ENV+ solid-phase cartridges, and the stacked cartridges were eluted sequentially with 1 mL of ethyl acetate 3 times. The sample was transferred to gas chromatography vials for instrumental analysis.

Instrumental Analysis. The extracted samples were analyzed by using a MDGC/MS (Agilent Technologies, Wilmington, DE) coupled with a PTV inlet, Gerstel MCS chromatographic system, and a multipurpose autosampler (Gerstel, Baltimore, MD). The MDGC/MS was composed of two Hewlett-Packard HP6890 Series Plus gas chromatographs (precolumn and analytical gas chromatograph) and a HP 5973 mass spectrometer (**Figure 1**). The mass spectrometer was coupled to the analytical gas chromatograph and operated using electron impact (EI) ionization under the scan mode.

Table 1. Recovery Data for Ethyl Carbamate in Wine at Three Different Concentrations

	ethyl carbamate recovery rate (%)	
concn (ng/mL) added	red wine ($n = 5$)	white wine ($n = 5$)
19.36	92.8	97.6
464.6	97.7	98.9
1006.7	99.6	99.2

The instrument was operated using a HP Chemstation data system under an NT operating system. The precolumn gas chromatograph (PGC) was a HP 6890 equipped with a flame ionization detector as the monitoring detector (*16*−*18*). A 35-μL aliquot of sample was injected at an injection speed of 0.6 μL/s. The inlet was operated under a split mode at an initial temperature of 4 °C and an initial time of 1.00 min. The temperature was then ramped at 10 °C/s to a final temperature of 270 °C and held for 3.00 min. The inlet was used in the solvent venting mode with a vent flow of 150 mL/min for 1 min (*19*−*23*). Chromatographic separation was performed with a 30 m × 320 μm i.d. HP-5MS (Agilent Technologies) fused-silica capillary column with a 0.25-μm film thickness. Helium was used as the carrier gas with the pressure set at 124.5 kPa and a total flow of 150 mL/min. The initial column temperature was set at 50 °C (held for 1.00 min) and was raised at a rate of 15 °C/min to 200 °C (held for 11 min). The switching valve that allowed the heart-cutting of the chromatogram was located in the PGC with a flow of 10 mL/min. A single heart-cut was performed from 4.8 to 5.5 min and transferred to a cryotrap located between the two gas chromatographs. The cryotrap was set with an initial temperature of −75 °C for 5.80 min. The temperature was then ramped at a rate of 20 °C/min to a final temperature of 240 °C (held for 7 min) to transfer the sample to the analytical gas chromatograph (AGC). Chromatographic separation was performed using a 30 m × 250 μm i.d. DB-WAX (Agilent Technologies) fused-silica capillary column coated with a 0.25-μm film thickness. The initial column temperature was set at 45 °C (held for 6.20 min) and was raised at a rate of 20 °C/min to 220 °C (held 5 min). The carrier gas flow into the mass spectrometer was controlled by Gerstel MCS system.

RESULTS AND DISCUSSION

This new approach to the analysis and quantitation of EC was undertaken to provide a simpler sample preparation

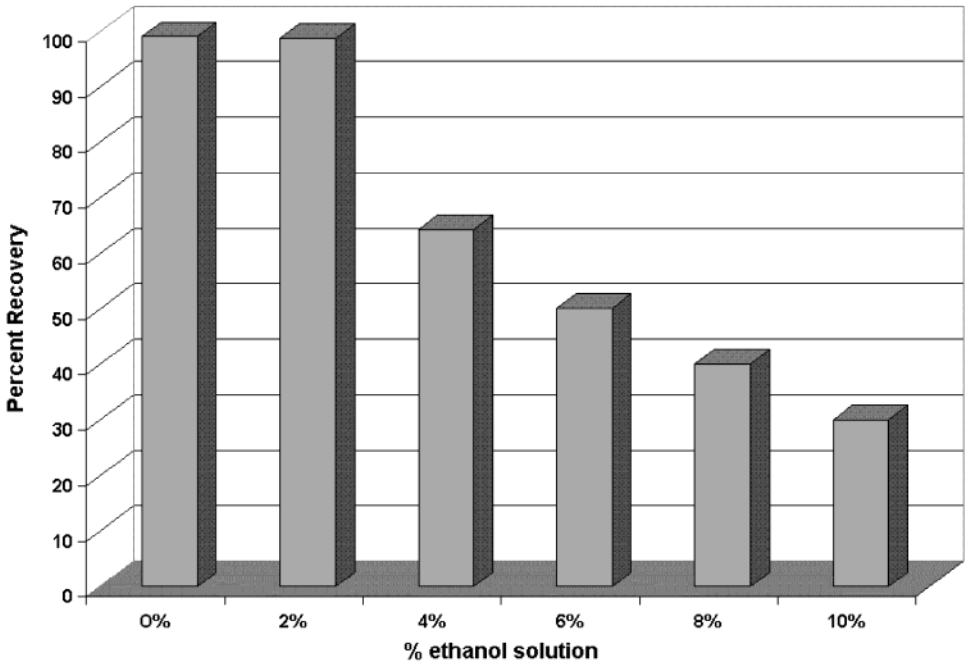

Figure 2. Percent recovery of EC in varying percent ethanol solutions for ENV+ sorbent.

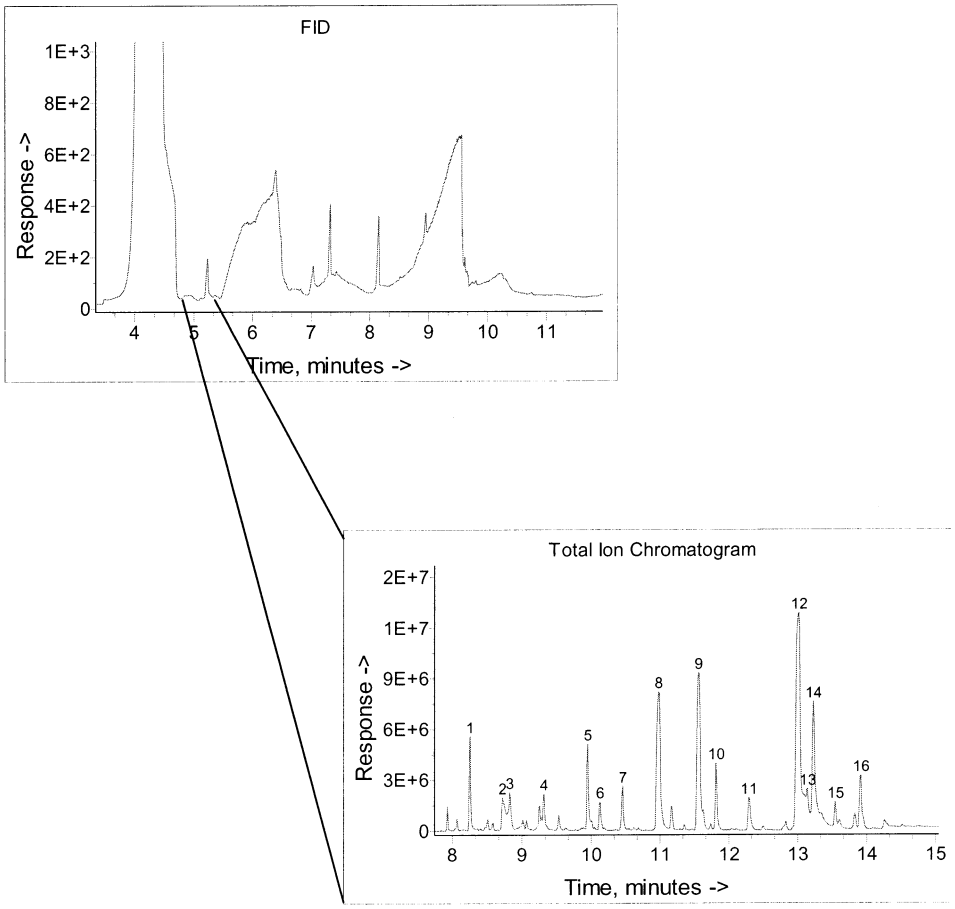

Figure 3. Heart-cutting of a standard of ethyl carbamate and isotopic labeled ethyl carbamate in wine using the multidimensional GC/MS. Peak 16 is the combined signal for the total ion chromatogram of ethyl carbamate and labeled ethyl carbamate.

technique than those currently used for a difficult matrix, such as wine. In developing a new solid-phase extraction method for the analysis of EC, one major obstacle encountered was the substantial amount of ethanol in the sample. For efficient solid-phase extraction, the ethanol had to be removed, because it acts to reduce the retention of EC on the ENV+ sorbent. A study of the amount of ethanol that could be present in the sample and not interfere with the retention of EC was undertaken. From

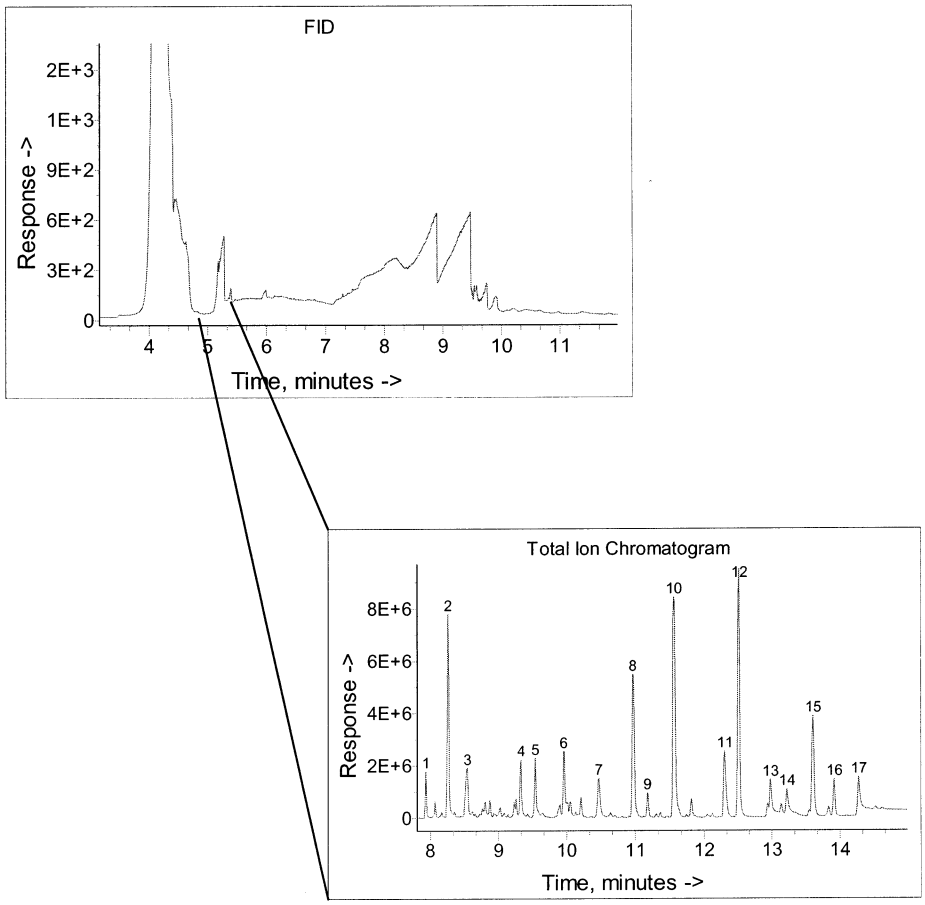

Figure 4. Ethyl carbamate extracted from a fruit/berry wine with labeled ethyl carbamate as the internal standard.

the graph, it demonstrated that the recovery of EC decreased as the percentage of ethanol in the sample increased (**Figure 2**). It was concluded that samples containing less than 4% ethanol were ideal for the ENV+ solid-phase material. Since wine samples contain more than 4% of ethanol, a new approach for the removal of ethanol was implemented. A centrifuge vacuum concentrator was used to remove the ethanol from the sample. Experiments were performed to verify that there was no loss of EC during the vacuum concentration process. In developing this method, three sorbents (ENV+, C_8, C_{18}) were evaluated for their retention of the compound of interest. Only ENV+ showed excellent results with percent recoveries >90% for samples with <4% ethanol content.

To validate the solid-phase extraction procedure, EC spikes were made at 19.36, 464.6, and 1007 ng/mL in wines. From the study, the white wine recovery at the 19.36 ng/mL was higher than the red wine, and this was attributed to the simpler sample matrix of white wine (**Table 1**). The intraday variability was determined for three replicates of quality control (QC) samples at each of the five calibration concentrations (19.36, 38.72, 77.44, 232.3, and 464.6 ng/mL) and assayed against a single calibration curve. The result shows the percent relative standard deviation (%RSD) was <2%. Likewise, the interday variation was evaluated by analysis of three replicates of QC samples (19.36, 38.72, 77.44, 232.3, and 464.6 ng/mL) on three separate occasions. The results show a %RSD of <6% for all of the levels.

The MDGC/MS system is a fairly new approach composed of two gas chromatographs and a mass spectrometer that allowed the use of two columns with their own temperature program rather than having both columns in one GC (*24*). This approach allows heart-cutting of peaks of interest and disposal of the unwanted portion of the chromatogram (**Figures 3** and **4**). The heart-cuts are passed to the cryotrap and subsequently to the analytical GC for further separation and identification by the mass spectrometer. The use of a labeled EC ($C_2{}^{13}CH_7O_2{}^{15}N$) was used as the internal standard, because it has the same retention time as EC, so only one heart-cutting was needed for this method. The mass spectrum of EC using EI ionization produced ions of *m*/*z* 89, 74, 62, and 44. The molecular ion at *m*/*z* 89 is weak and not ideal for quantitation. The mass spectrum of the labeled EC contains corresponding ions of *m*/*z* 91, 76, 64, and 46 (**Figure 5**). These ions are two mass units more than unlabeled EC because of the labeled nitrogen and labeled carbon. The ions at *m*/*z* 62 (EC) and 64 (labeled EC) were chosen for quantitation because they are susceptible to fewer interferences. The fragments at *m*/*z* 44 and 74 are common to interference from background and alkyl methyl esters (*25*).

A linear dynamic range was established from 0.387 to 1160 ng/mL, with a correlation coefficient of >0.9999. The limit of detection was determined to be 0.1 ng/mL, and the limit of quantitation was determined to be 1 ng/mL using the signal-to-noise ratio of 10:1.

For sample analysis, no particular type of sample was targeted. All samples that were available to the agency were screened using this method (**Table 2**). Further studies will be designed to do a thorough screening of a wide variety of

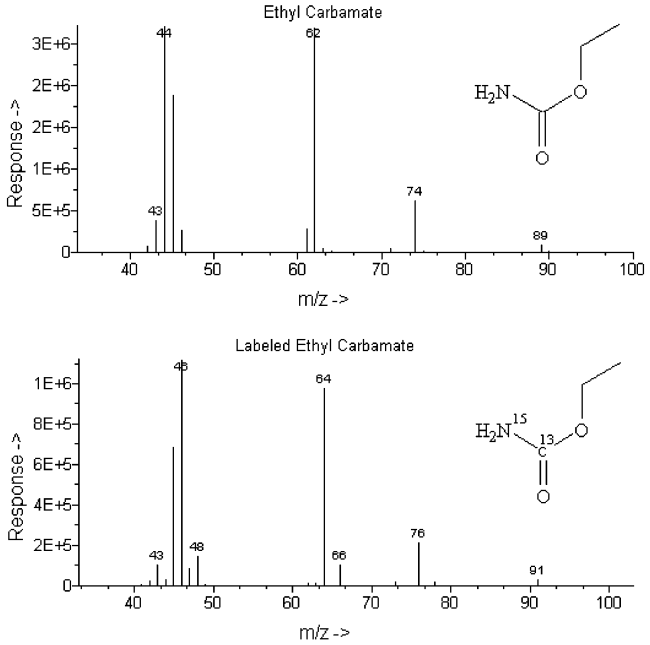

Figure 5. Mass spectra of ethyl carbamate and labeled ethyl carbamate.

Table 2. Concentration of Ethyl Carbamate Detected in Wine Samples

	concn detected (ng/mL)		
samples	range	av	count (*n*)
table wine, red	54–n.d.[a]	12	67
table wine, white	19–n.d.	8	69
port wine	50–14	30	8
sherry-type wine	58–n.d.	31	6
vermouth/aperitif/flavored wine	112–n.d.	31	13
fruit/berry wine	114–n.d.	86	4
sake/rice wine	125–n.d.	111	19

[a] n.d.= not detected.

samples. The results indicate that fruit/berry wines and sake samples were high. From a 10-year study of samples containing high EC using other methods, these products are on the list.

CONCLUSION

The method employed to determine the amount of EC in wines has shown several advantages. The use of a solid-phase extraction procedure, followed by MDGC/MS method is cost-effective, environmentally friendly, and efficient for the analysis of EC in wines. This method eliminates the use of large volumes of methylene chloride that is being employed by other methods. The validation procedure has established that the described method for the analysis of EC offers excellent reproducibility, precision, and accuracy over a wide dynamic linear range.

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