RAPID COMMUNICATIONS IN MASS SPECTROMETRY
Rapid Commun. Mass Spectrom. 2005; **19**: 108–112
Published online in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/rcm.1755

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Application of tandem mass spectrometry combined with gas chromatography to the routine analysis of ethyl carbamate in stone-fruit spirits

Dirk W. Lachenmeier*, Willi Frank and Thomas Kuballa

Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, Weißenburger Str. 3, D-76187 Karlsruhe, Germany

Received 21 September 2004; Revised 3 November 2004; Accepted 3 November 2004

Gas chromatography (GC) coupled to mass spectrometry (MS) operated in selected ion monitoring (SIM) mode is currently the method of choice for the determination of the toxic contaminant ethyl carbamate in alcoholic beverages. However, even after extensive sample cleanup over diatomaceous earth columns, the identity of ethyl carbamate often cannot be ascertained with confidence, due to inconsistent ratios of the SIM ions *m/z* 62, 74 and 44 because the qualifier ions are highly susceptible to interferences. Therefore, a new method combining GC and tandem MS using a triple-quadrupole instrument is introduced to determine ethyl carbamate in stone-fruit spirits. For quantitative analysis the characteristic transitions of *m/z* 74 → 44 and *m/z* 62 → 44 for ethyl carbamate as well as *m/z* 64 → 44 for the deuterated internal standard ethyl carbamate-d_5 were monitored in the multiple reaction monitoring (MRM) mode. In the validation studies, ethyl carbamate exhibited good linearity with a regression coefficient of 1.000. The limits of detection and quantitation were 0.01 and 0.04 mg/L. The recovery of the method was 100.4 ± 9.4%. The precision never exceeded 7.8% (intraday) and 10.1% (interday) and the trueness never exceeded 11.3% (intraday) and 12.2% (interday) at any of the concentrations examined, indicating good assay accuracy. A good agreement of analytical results between a previously developed GC/MS SIM method and the GC/MS/MS MRM procedure was found (R = 0.987). Regarding the validation data, the procedure is sensitive, selective and reproducible. The applicability of the developed method was demonstrated by the investigation of 70 stone-fruit spirits from commercial trade. The ethyl carbamate concentration of the samples ranged between 0.07 and 7.70 mg/L (mean 1.21 mg/L). The main advantage of the developed GC/MS/MS method is the reliability of the results without the need for time-consuming confirmatory analyses.

Ethyl carbamate (urethane, $C_2H_5OCONH_2$) is a known genotoxic carcinogen of widespread occurrence in fermented food and beverages.[1–5] Public health concern concerning ethyl carbamate in alcoholic beverages began in 1985 when relatively high levels were detected by Canadian authorities.[6] The highest ethyl carbamate concentrations were found in spirits derived from stone fruits (e.g. cherries, plums, mirabelles, apricots).[2,3] Subsequently, Canada established an upper limit of 0.4 mg/L ethyl carbamate for fruit spirits,[6] which was adopted by many other countries.

Cyanide formed by enzymatic action and thermal cleavage of cyanogenic glycosides such as amygdalin in stone fruits is the most important ethyl carbamate precursor in spirits. Cyanide is oxidised to cyanate, which reacts with ethanol to form ethyl carbamate.[2,7–9] The wide range of ethyl carbamate concentrations in stone-fruit spirits reflects its light-induced and time-dependent formation after distillation.[3,10–13]

Many preventive actions to avoid ethyl carbamate formation in alcoholic beverages have been proposed. Among these are the addition of patented copper salts to precipitate cyanide in the must,[14,15] distillation using copper catalysts,[16,17] or the application of steam washers.[18] Despite the information available to distilleries about the ethyl carbamate problem, 30% of the analysed products (1996–2002) exceed the upper limit by more than a factor of 2. Small distilleries that have not introduced improved technologies especially have this problem. Therefore, efficient routine methods for the determination of ethyl carbamate in spirit drinks are needed in food control.

Gas chromatography coupled with mass spectrometry (GC/MS) seems to be the method of choice for the analysis of ethyl carbamate in alcoholic beverages.[3,6,12,19–28] The overwhelming majority of these procedures involves a quadrupole mass spectrometer operating in selected ion monitoring (SIM) mode. However, the analysis of minor organic

**Correspondence to*: D. W. Lachenmeier, Chemisches und Veterinäruntersuchungsamt (CVUA) Karlsruhe, Weißenburger Str. 3, D-76187 Karlsruhe, Germany.
E-mail: lachenmeier@web.de

compounds in complex matrices like spirit drinks is difficult because of interferences by matrix components, even when extensive cleanup procedures are applied to the sample, e.g. extraction over diatomaceous earth columns proposed by many authors.[12,29–34] On the one hand, a possible approach to eliminate these interferences is the use of solid-phase extraction (SPE) in combination with an improved chromatographic separation using multidimensional GC, as proposed by Jagerdeo *et al*.[27] for wine analysis. However, this technique requires the time-consuming removal of ethanol before SPE, and specialised equipment consisting of a gas chromatograph with flame ionisation detector and a GC/MS system, which are coupled using a cryo trap. On the other hand, the mass spectrometric detection may be enhanced as presented in this study. The use of GC coupled to tandem mass spectrometry (MS/MS) using triple-quadrupole mass spectrometers, providing an improved sensitivity and specificity, has been demonstrated in the analysis of wine and grain spirits.[35] For a long time this technology was restricted to expensive instruments, and only used to provide structural confirmation of samples that were positive in conventional GC/MS. In the analysis of spirit drinks, a lack of accuracy and precision in the collision-induced dissociation (CID) of ethyl carbamate was reported.[36] Recently, the introduction of low-cost benchtop triple-quadrupole mass spectrometers made it possible to adopt these techniques in routine analysis, e.g. in forensic hair analysis.[37] The Kodiak 1200 MS/MS system uses a 180° curved collision cell, which also positions the electron multiplier off-axis from the source for lower background noise. To evaluate this technique, the mass spectrometer was operated in multiple reaction monitoring (MRM) mode and results were compared with those from a previously developed GC/MS SIM method that has been used in routine analysis since 1986.[12] In this study, GC/MS/MS was applied for the first time to routine analysis of ethyl carbamate in stone-fruit spirits.

EXPERIMENTAL

Chemicals

Ethyl carbamate and ethanol-d_6 were purchased from Sigma-Aldrich (Taufkirchen, Germany). Extrelut NT 20 columns, Extrelut NT 20 refill material, as well as aluminium oxide 90 (Brockmann-activity level II) and trichloroacetyl isocyanate, were obtained from Merck (Darmstadt, Germany).

Synthesis of deuterated ethyl carbamate

As ethyl carbamate-d_5 for use as an internal standard was not commercially available, synthesis was carried out according to Kočovský[38] with modifications according to Funch and Lisbjerg.[19] Trichloroacetyl isocyanate (2.77 g) diluted in dichloromethane (3 mL) was placed in a 10-mL test tube and cooled in an ice bath. Ethanol-d_6 (1.0 mL) was added dropwise. The solution was left under nitrogen for 15 min at room temperature and then was transferred into a sintered-glass filter funnel filled with approximately 15 g of aluminium oxide 90. After 15 min, the reaction product was washed out with toluene/dichloromethane (3×15 mL; $2 + 1$, v/v) and was carefully dried using a rotary evaporator. The crystals were colourless and ice-like. Yield: 1.44 g (90%). EI-MS: (m/z) (rel. abundance.): 44 (100), 64 (84.6), 76 (16.5).

Instrumentation

The GC/MS/MS system used for analysis was an Agilent model 6890 Series Plus gas chromatograph in combination with a CTC Combi-PAL autosampler and a Bear Instruments Kodiak 1200 MS/MS triple-quadrupole mass spectrometer (Chromtech, Idstein, Germany). Data acquisition and analysis were performed using standard software supplied by the manufacturer (Kodiak Software 2.1.023 and CTC Cycle Composer 1.5.2). Substances were separated on a fused-silica capillary column (CP-wax, 49 m × 0.25 mm i.d., film thickness 0.25 μm). Temperature programme: 50°C hold for 1 min, 5°C/min up to 160°C, hold for 0 min, 25°C/min up to 220°C, hold for 10 min. The temperatures for the injection port, ion source and transfer line were set at 220, 200 and 280°C, respectively. Splitless injection mode (1.5 min) was used and helium with a constant flow rate of 1.0 mL/min was used as carrier gas. MS/MS experiments were based on CID occurring in the collision cell (quadrupole 2) of the triple quadrupoles, with an argon collision gas pressure of approximately 2.0 mTorr and an offset voltage of −20 eV.

To determine the retention times and characteristic mass fragments, the primary electron ionisation (EI) mass spectra and the product spectra of the analytes were recorded in full-scan mode (m/z 35-100). For quantitative analysis the chosen fragmentations were monitored in the multiple reaction monitoring (MRM) mode: m/z 74 → 44 and m/z 62 → 44 for ethyl carbamate and m/z 64 → 44 for ethyl carbamate-d_5 as the internal standard. For quantification, peak area ratios of the analytes to the internal standard were calculated as a function of the concentration of the substances.

Samples and sample preparation

Stone-fruit spirit samples were submitted by local authorities to the CVUA Karlsruhe for analysis. Our institute covers the district of Karlsruhe in North Baden (Germany), which has a population of approximately 2.7 million and includes the northern part of the Black Forest, a territory with around 14 000 approved distilleries producing well-known specialties like Black Forest Kirsch (cherry spirit).

The sample preparation was previously optimised for conventional mass spectrometric determinations[12] using a modified procedure of Baumann and Zimmerli.[29] Volumes of 20 mL of stone-fruit spirit were spiked with 50 μL of ethyl carbamate-d_5 (1 μg/mL) and directly applied to the extraction column filled with one Extrelut package mixed with 10 g of sodium chloride. The Extrelut column was wrapped in aluminium foil to eliminate the possibility of ethyl carbamate formation during extraction. After 15 min of equilibration, the column was washed with 2×20 mL of n-pentane. Next, the analytes were extracted using 3×30 mL of dichloromethane. The eluates were combined in a brown flask and reduced to 2–3 mL in a rotary evaporator (30°C, 300 mbar). After that, the solution was adjusted to 10 mL with ethanol in a measuring flask and directly injected into the GC/MS/MS system. In addition, to evaluate the light-induced ethyl carbamate formation capability of the products, all samples were exposed to UV light for 4 h using a Psorilux 3060 lamp

(Heraeus, Hanau, Germany) and extracted as described above.

Validation studies

For the validation of the method, three authentic samples with varying alcohol and ethyl carbamate contents were extracted and analysed several times intraday (n = 5) and interday (n = 10). The linearity of the calibration curves was evaluated between 0.25 and 5 mg/L. For the determination of the limit of detection (LOD) and the limit of quantitation (LOQ) a separate calibration curve in the range of LOD (0.01–0.1 mg/L) was established.[39,40] For the determination of the recovery, samples were spiked with 1 mg/L of ethyl carbamate.

RESULTS AND DISCUSSION

Single-stage mass spectrometry, using electron impact ionisation and selected ion monitoring according to the method of Mildau *et al.*,[12] has been successfully used for many years in our laboratory. The mass spectrum of ethyl carbamate shows only a weak molecular ion at *m/z* 89, $[M]^+$, and a relatively weak fragment at *m/z* 74, $[M-CH_3]^{+\cdot}$. It is further dominated by fragment ions at *m/z* 44 $[NH_2CO]^+$, *m/z* 45 $[C_2H_5O]^+$ and, especially, the resonance-stabilised ion at *m/z* 62 $[M-C_2H_3]^+$, which derives from a 'McLafferty + 1' rearrangement.[28,35] The ions at *m/z* 62 (ethyl carbamate) and *m/z* 64 (ethyl carbamate-d_5) were used for quantitation because they are characteristic for the carbamate structure, have the highest relative abundance, and were found to be insusceptible to interferences.[12,27,28,32] The selection of qualifier ions posed more of a problem: the molecular ion is not suitable because of its low abundance. The fragment ions at *m/z* 44, 74 and 76 were frequently superimposed by interferences, even if multidimensional gas chromatographic techniques were used.[27] In particular, *m/z* 44 is highly susceptible to chemical background (e.g. carbon dioxide) and is yielded by both ethyl carbamate and its deuterated analogue. The ion at *m/z* 74 is a common ion for all alkyl methyl esters and often shows interferences, even after extensive sample cleanup.[12,28,32] For pragmatic reasons the fragments at *m/z* 74 and 44 were chosen as qualifiers;[12] however, the identity of ethyl carbamate often could not be ascertained with confidence due to inconsistent ratios of the three ions. In these cases, a time-consuming verification of identity by standard addition had to be carried out, especially to fulfil the increasing demands concerning the performance of analytical methods in official food monitoring.[41] In order to study the fragmentation pattern of ethyl carbamate, MS/MS product-ion experiments were performed. For the generation of product spectra, the base peak at *m/z* 62 and qualifier peak at *m/z* 74 were chosen. The fragmentation of the precursor ion is performed in the second quadrupole by CID with argon gas in combination with an additional collision cell offset voltage. The product spectra were evaluated in the third quadrupole in full-scan mode to determine the most abundant product ion. Product-ion mass spectra of ethyl carbamate are reported in Figs. 1(a) and 1(b) as an example of the spectral quality obtained. After that, the fragmentation reaction of the chosen precursor/product-ion pair was optimised by varying the

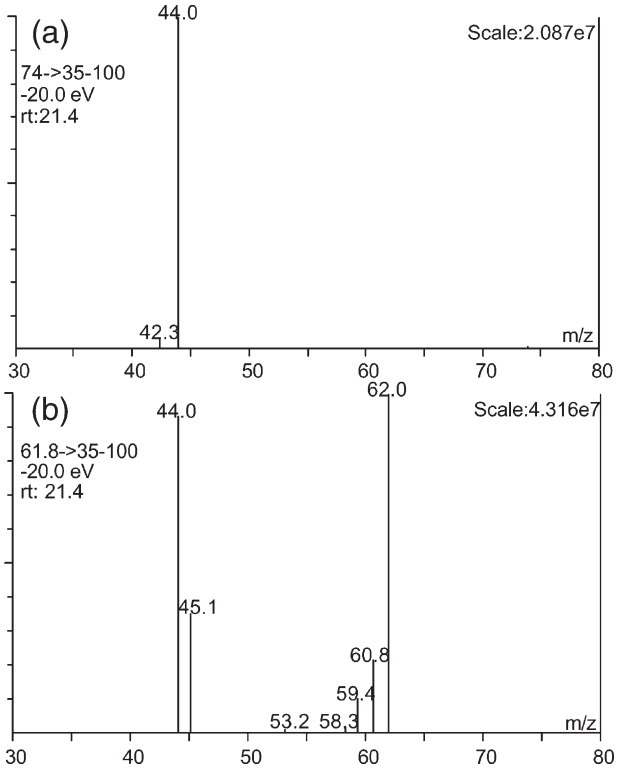

Figure 1. Positive-ion MS/MS product-ion mass spectra of ethyl carbamate: *m/z* 74 (a) and *m/z* 62 (b).

offset voltage between −5 and −20 eV. The optimal fragmentation reactions were *m/z* 74 → 44 ($[M-CH_3-CH_2O]^+$) and *m/z* 62 → 44 ($[M-C_2H_3-H_2O]^{+\cdot}$) at −20 eV.

For comparison, authentic samples were analysed using both the GC/MS SIM and the GC/MS/MS MRM modes. Figure 2(a) shows a chromatogram obtained in SIM mode. The peaks were embedded in matrix peaks. In Fig. 2(b) a chromatogram for the identical sample analysed using MRM is shown. Comparing the chromatograms, the superiority of MRM was obvious. Distinct peaks appeared for ethyl carbamate and ethyl carbamate-d_5 with small or no impurity peaks. By analysing 70 authentic samples, the interferences observed in SIM mode using conventional mass-selective detectors were removed.

The relative signal intensity of the transition *m/z* 62 → 44, which was used for quantification, to the qualifier transition *m/z* 74 → 44 corresponded in all samples within a tolerance of ±10% to that of the calibration standard solutions, thus confirming the identity of the analyte. Obviously, MRM gave higher sensitivity and selectivity than the SIM mode for the determination of ethyl carbamate in stone-fruit spirits. Therefore, MRM was used for further experiments.

In the validation studies, ethyl carbamate exhibited good linearity with a regression coefficient of 1.000. The limits of detection and quantitation were 0.01 and 0.04 mg/L, respectively, being over 10 times lower than the limits of the corresponding GC/MS SIM procedure. The limit of detection attained by GC/MS/MS was comparable with that of the method of Cairns *et al.*[36] The sensitivity of the method is, therefore, adequate to check the upper limit of 0.4 mg/L in stone-fruit spirits. Higher sensitivity, e.g. required for wine analysis, may be achieved using the improved sample

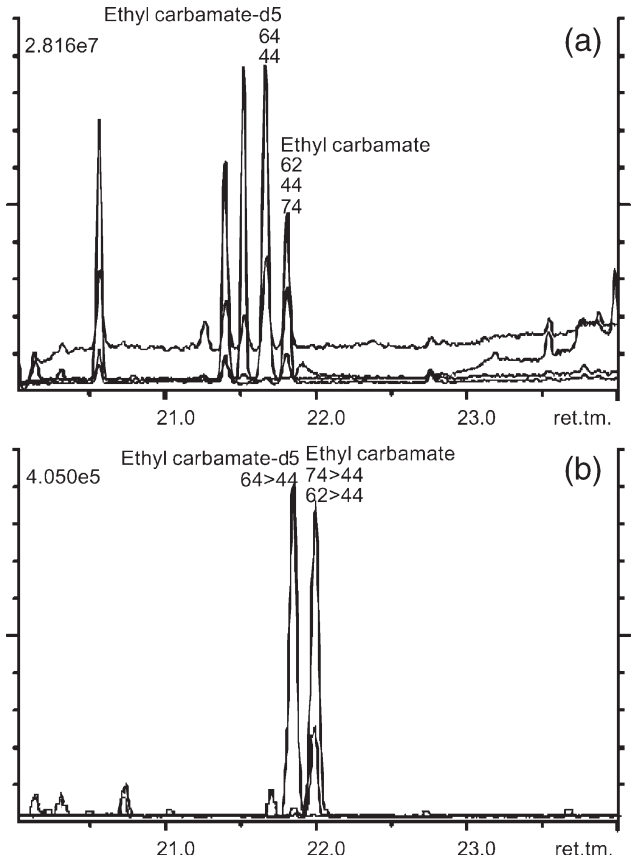

Figure 2. Positive-ion GC/MS SIM chromatogram (a) in comparison with the corresponding GC/MS/MS MRM chromatogram (b) of an authentic mirabelle spirit containing 0.63 mg/L of ethyl carbamate.

preparation and multidimensional chromatographic separation of Jagerdeo *et al*.[27]

The recovery of the GC/MS/MS method was 100.4 ± 9.4%. Table 1 summarises the results of method accuracy studies. As a result of the use of a deuterated internal standard in combination with MRM, the precision never exceeded 7.8% relative standard deviation (RSD) (intraday) and 10.1% (interday) and the trueness never exceeded 11.3% (intraday) and 12.2% (interday) at any of the concentrations examined, indicating good assay accuracy. No lack of accuracy in the CID of ethyl carbamate, as reported in early GC/MS/MS experiments,[36] was observed during our study.

Good agreement of analysis results between the previously developed GC/MS SIM method[12] and the GC/MS/MS MRM procedure was found. The linear regression equation was $y = 0.96 \pm 0.03\,x + 0.05 \pm 0.05$. The linearity of the correlation between the two methods was significant ($p < 0.0001$), with a coefficient of correlation of 0.987. The confidence interval was 0.90 to 1.03 for the slope and −0.04 to 0.15 for the y intercept. Since slope and intercept encompass the theoretical values, no constant or proportional difference between the two procedures could be proven other than random errors.

Regarding the validation data, the procedure is sensitive, selective and reproducible. The applicability of the developed method was demonstrated by the investigation of 70 food samples from commercial trade. The ethyl carbamate concentration of the samples ranged between 0.07 and 7.70 mg/L (mean 1.21 mg/L). After exposure of the samples to UV light, significantly ($p = 0.001$) higher concentrations, between 0.08 and 8.81 mg/L (mean 1.74 mg/L), were determined. The ethyl carbamate concentration increased on average by 0.57 ± 1.31 mg/L. Official complaints had to be made against 26 distilleries (37%), because their products exceeded the upper limit of 0.4 mg/L by more than a factor of 2. However, in official food control, lot-to-lot differences and inhomogeneities have to be considered. Therefore, the manufacturers were advised of their duty to exercise diligence and to use the state-of-the-art measures needed to reduce the content of the contaminant, ethyl carbamate.

CONCLUSIONS

The results show that nearly 20 years after the first warnings about ethyl carbamate in spirit drinks, the problem persists. GC/MS/MS is an efficient technique that can be used for the identification of organic compounds present at trace levels in food samples. Triple-quadrupole mass spectrometry appears ideal for the quantification of small amounts of contaminants in complex matrices over a wide concentration range, particularly in the field of food analysis. Even analyses using cleanup procedures can be significantly improved by MS/MS. Due to a decrease in the acquisition costs of benchtop triple-quadrupole mass spectrometers, GC/MS/MS will probably be the successor to GC/MS SIM technology for the analysis of contaminants in food matrices in the near future. The developed GC/MS/MS method yields reliable results without the need for time-consuming confirmatory analyses like standard addition. Therefore, the efficiency of the procedure is superior to conventional ones and the number of samples may be increased to produce better consumer protection.

Table 1. Accuracy of the GC/MS/MS method determined using authentic cherry spirits with different ethanol and ethyl carbamate concentrations

		Intraday (n = 5)		Interday (n = 10)	
Ethanol [%vol]	Ethyl carbamate [mg/L]	Precision[a] [%]	Trueness[b] [%]	Precision[a] [%]	Trueness[b] [%]
42	1.56	4.7	4.8	10.1	5.8
40	0.49	7.8	11.3	7.9	12.2
38	1.26	7.3	−3.8	8.3	−4.0

[a] Precision is expressed as RSD [%].
[b] Trueness is expressed as bias (difference between GC/MS SIM and GC/MS/MS [%]).

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Acknowledgements

The authors thank S. Gonzalez, H. Heger and H. Havel for excellent technical assistance. Presented at the 33rd Deutscher Lebensmittelchemikertag (Bonn, Germany).

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