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Gas chromatographic–mass spectrometric determination of ethyl carbamate as the xanthylamide derivative in Italian aqua vitae (grappa) samples

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ABSTRACT

A selective reaction of ethyl carbamate (urethane) and methyl urethane (urethylane), as internal standard, with xanthydrol was effected to detect urethane after extraction from Italian aqua vitae (grappa) samples. The xanthylamides formed were determined by gas chromatography–mass spectrometry in the selected ion monitoring mode on an apolar DB 5 silica column. The linearity of the method was tested from 10 to 1000 μg/l, with a detection limit of 1 μg/l.

INTRODUCTION

Initial studies on urethane in alcoholic beverages indicated that it is formed as a result of a reaction between diethyl pyrocarbonate (DEPC) and beverages containing naturally occurring amounts of ammonia [1]. DEPC had been widely used as an antimicrobial additive until 1972. Successive studies by Ough [2–4], however, demonstrated that urethane occurred naturally in fermented foods and beverages and that the addition of DEPC to wines minimally affected the amount of urethane produced.

As urethane is carcinogenic [5], it became essential to ensure that products for human consumption, including alcoholic beverages, contained minimum levels of the substance. In 1986 the Department of Health and Welfare in Canada issued [16] guidelines limiting the levels of ethyl carbamate in wines, distilled spirits, fruit brandies and liqueurs.

Reported urethane assays are based on gas chromatographic (GC) separation and detection with Hall electrolytic conductivity detectors in the nitrogen mode [6–9]. In conjunction with this techniquc, mass spectrometric (MS) detection was used for confirmation or for direct quantitative evaluations [10–12]. Urethane has also been determined by GC using nitrogen–phosphorus thermionic detection after a methylation reaction [13] or using two-dimensional GC and flame ionization detection [14].

This paper describes a further assay procedure based on a selective reaction of urethane with xanthydrol and GC–MS measurement of the xanthylamide formed. The method was applied to the determination of urethane in twenty samples of the Italian grape distillate aqua vitae (grappa). The results obtained (values ranging from 70 to 400 μg/l) confirmed the sensitivity and accuracy of the method.

EXPERIMENTAL

Chemicals

Xanthydrol, urethane, urethylane and the solvents used, all of analytical-reagent grade, were supplied by Fluka (Buchs, Switzerland).

Authentic xanthylurethane and xanthylurethylane were synthesized by dissolving 250 mg of xanthydrol in 3 ml of glacial acetic acid with 250 mg of either ethyl carbamate or methyl carbamate, gently warmed in a water-bath at 37°C. The resulting solutions were allowed to stand for 20 min in the same bath, then cooled to obtain crystals. These were re-

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crystallized from dioxane–water (1:1, v/v) and dried at 70°C for 15 min [15].

The following solutions were prepared: 0.1 mg/ml xanthydrol in glacial acetic acid, 0.1 and 0.01 mg/ml urethane in ethyl acetate and 0.01 mg/ml urethylane in ethyl acetate.

Extraction procedure

The grappa sample (10 ml) was washed twice with *n*-pentane (5 ml), vortex mixed for 1 min and the solvent discarded each time. After addition of sodium chloride (200 mg) and urethylane standard solution (25 μl), the sample was extracted with dichloromethane–ethyl acetate (95:5, v/v) (10 ml) by vortex mixing for 20 s and shaking for 10 min. The sample was then briefly centrifuged at 625 *g*, the organic phase transferred to another tube containing anhydrous sodium sulphate and the aqueous phase re-extracted twice with dichloromethane–ethyl acetate (90:10, v/v) (5 ml). All the dehydrated extracts were evaporated under a stream of nitrogen in a water-bath at 37°C until the volume was reduced to 10–20 μl. Xanthydrol standard solution (0.1 ml), glacial acetic acid (0.1 ml) and water (0.3 ml) were then added to this solution. After mixing, the sample was left in a warm bath (37°C) for 20 min, cooled with a further 0.6 ml of ice-cold water and re-extracted with dichloromethane–ethyl acetate (90:10, v/v) (5 ml) by shaking for 5 min. After centrifugation, the organic layer was removed and evaporated to dryness under nitrogen. The residue was redissolved in two drops of ethyl acetate, mixed with 50–100 μl of *n*-hexane and 1 μl was injected for analysis.

Quantification

Standard amounts of urethane in the range 10–1000 ng were added to tubes containing 10 ml of ethanol–water (50:50, v/v). A 250-ng amount of urethylane was added as internal standard and then the mixture was subjected to the extraction procedure and reaction with xanthydrol as described above. Quantification was based on the peak-area ratios of xanthyl urethane to xanthyl urethylane *versus* added amounts. The resulting calibration graphs and linear regressions are shown in Fig. 1. Each point is the mean value from triplicate analyses.

Gas chromatography–mass spectrometry (GC–MS)

A Hewlett-Packard quadrupole mass spectrometer (Model 5970 B) connected with a Carlo Erba HRGC 5300 Mega Series gas chromatograph was used. GC was performed on a 15 m × 0.24 mm I.D. fused-silica capillary column coated with DB 5 (film thickness 0.25 μm) (J&W Scientific, Folsom, CA, USA) preinserted in a retention-gap silica column (1 m). The oven was programmed from 150°C (held for 1 min) to 180°C at 30°C/min, then to 205°C at 3.5°C/min, and finally to 260°C (held for 5–10 min) at 30°C/min. The injector and interface were maintained at 250°C. The sample injection was splitless

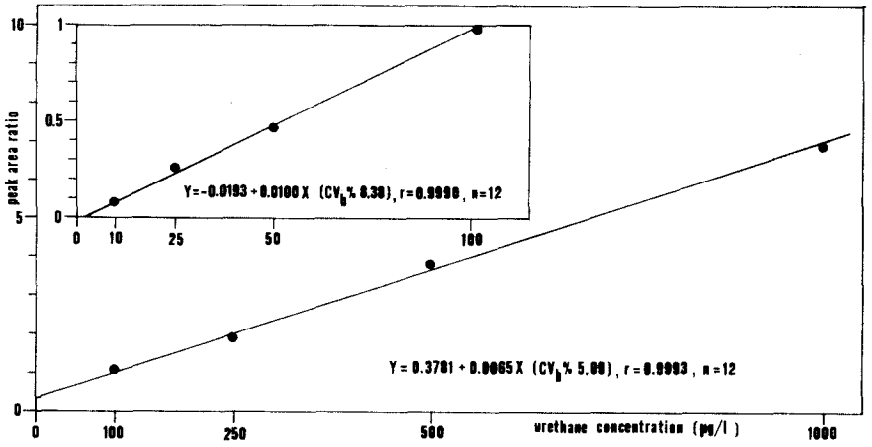

Fig. 1. Calibration graphs for the determination of urethane in grappa samples.

(30 s), splitting ratio 1:10. The carrier gas was helium at a flow-rate of 1.5 ml/min. Mass spectra were obtained in the scanning mode using positive-ion electron impact (EI) ionization (70 eV). Data processing on selected ion monitoring (SIM) mode acquisition was performed on a Hewlett-Packard 59970 C Chem Station.

Data acquisition

The peak areas of xanthylurethane or xanthylurethylane were obtained from the peaks at the *m/z* 76, 77, 90, 152, 181, 182, 195, 196, 197, 222, 240, 241, 255 and 269 in the mass chromatogram with SIM mode acquisition, using a time window from 6 to 8 min. A 100-μs dwell time was applied to each ion monitored. The resulting voltage was 1.8 kV and the scan time was 0.6 cycles per second. Mass calibration was relative to external lock, from perfluorotributylamine.

RESULTS AND DISCUSSION

One of the reactions in organic chemistry that can be used to identify amides is that with xanthydrol. In fact, we easily succeeded in obtaining high yields of xanthylamides (>95%) by reacting urethane and urethylane with an excess of reagent (the molar equivalent ratios being 1:2.22 and 1:2.64, respectively). The crystalline products obtained when analyzed by GC–MS gave rise to the fragmentation spectra shown in Fig. 2.

Under electron impact both xanthylurethane and xanthylurethylane undergo a common fragmentation involving the alkylamide side-chains (Fig. 3). The high relative intensities of the *m/z* 196 and 181 peaks suggest a possible direct cleavage of the amide bond (M − 59, M − 73) with further loss of the remaining amino group. Alternatively, fragmentation might proceed stepwise through dealkylation (M − 29, M − 15) followed by CO_2 loss. Interestingly, the presence of the *m/z* 152 peak could be explained by the highly stable structure proposed in Fig. 3 which may be derived from *m/z* 181 by the loss of formaldehyde. The use of methylurethane (urethylane) as a suitable internal standard was suggested by the fact that the product is not a natural constituent of alcoholic beverages nor is it formed during their distillation process, and it retains the same chemical characteristics as urethane.

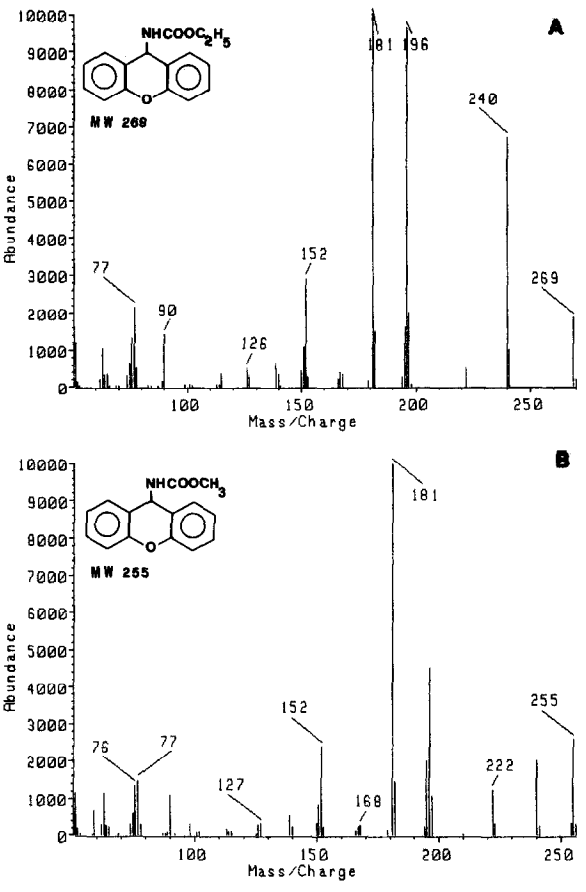

Fig. 2. GC–EI-MS of (A) xanthylurethane and (B) xanthylurethylane. MW = Molecular weight.

The application of such a highly selective reaction and detection method to the determination of urethane in grappa samples provided several advantages. The reaction products are simple to obtain, easily extractable and are more stable at high temperature than the underivatized compounds (urethane, m.p. 48°C; xanthylurethane, m.p. 169°C; urethylane, m.p. 54°C; xanthylurethylane, m.p. 193°C). As a consequence, xanthylamides do not decompose in the injection port and are not adsorbed on active sites such as on a contaminated liner, as could happen with other co-extracted polar compounds. The increased lipophilicity and thermal stability of the two derivatives permit both the use of a short apolar column, which reduces the analysis times, and the selection of a higher temperature

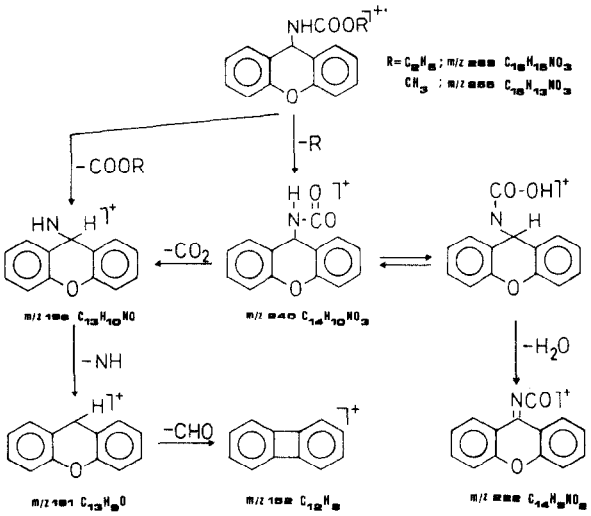

Fig. 3. Proposed fragmentation pattern of the xanthyl derivatives of urethane and urethylane.

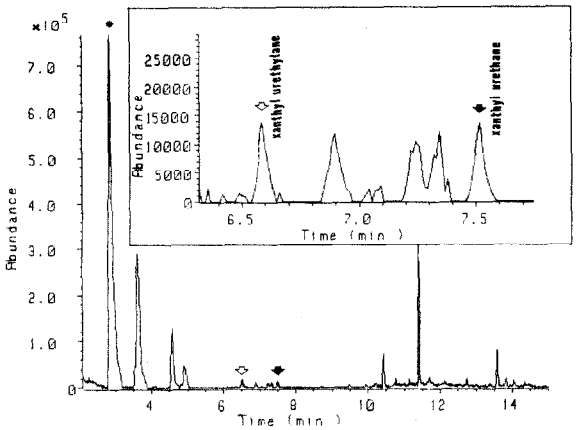

Fig. 4. Total ion current chromatogram (scan mode acquisition from m/z 50 to 280) for qualitative analysis of an aqua vitae (grappa) sample, extracted, derivatized and further spiked with xanthylamides standard. The peak marked with an asterisk is unreacted xanthydrol.

to elute the compounds in a relatively free zone of possible interfering peaks.

In the extraction procedure, dichloromethane–ethyl acetate was extremely effective (the absolute recoveries ranged from 85% to 95%) in recovering urethane and urethylane from aqueous ethanol solutions, which could be considered as equivalent to distilled spirit, so that large amounts of solvents were not needed and minimal analytical apparatus was used. In addition, the washing with *n*-pentane was effective in eliminating heavy compounds such as waxes and esters usually present in these samples.

The final results obtained can be exemplified by the chromatograms depicted in Fig. 4, recorded for qualitative evaluations (scan mode), and Fig. 5, effected with SIM mode acquisition for quantitative analysis. The linearity from 10 to 100 and from 100 to 1000 μg/l (Fig. 1) was tested and intra- and inter-assay tests were performed. At the two concentrations chosen (100 and 500 μg/l), the intra-assay test, carried out on ten samples analysed in one day, showed average recoveries of 95.4% and 98.6% with relative standard deviations (R.S.D.s) of 12.5% and 4.9%, respectively. Similarly, three samples a day for 10 days were analysed for the inter-assay test, obtaining average recoveries of 94.1% and 103.5% with R.S.D.s of 14.7% and 6.6%, respectively.

Concentrations of μg/l were considered presumptive evidence for the presence of urethane, even though the sensitivity could be increased to 1 μg/l when only the m/z 181 ion is considered (signal-to-noise 2:1). Under the above conditions, the urethane concentration found in 20 grappa samples ranged from 70 to 400 μg/l. Excluding some samples from batches of home-made grappa, the average value was 87 μg/l, which is consistent with the

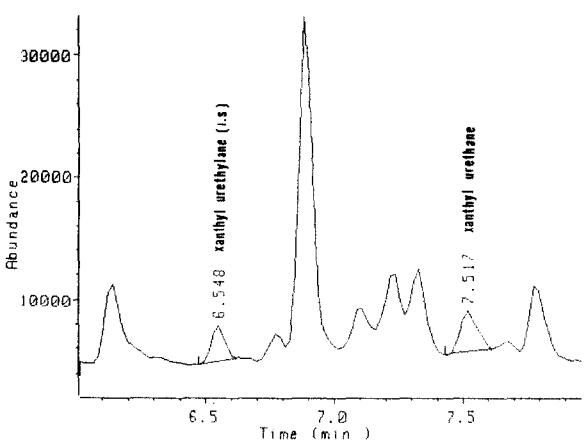

Fig. 5. Typical chromatographic trace (SIM mode acquisition) for the determination of urethane in an aqua vitae (grappa) sample extracted and derivatized. The amount found is 130 μg/l.

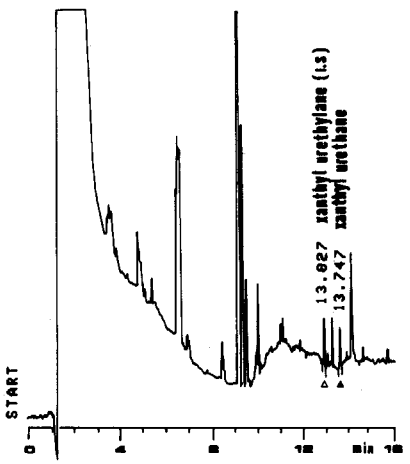

Fig. 6. GC profile of an aqua vitae (grappa) sample, treated as described in the text, with nitrogen–phosphorus detection using a 30 m × 0.24 mm I.D. DB 5 fused-silica capillary column (film thickness 0.25 μm). The amount of urethane found is 78 μg/l.

results obtained by other laboratories (range 7–360 μg/l) with validated GC–MS methods.

The urethane levels found in the home-made batches were higher than those in samples from distillery companies, thus confirming the hypothesis that inadequate temperature control during the distillation process increases urethane formation.

Finally, some standards and analytes were analysed using nitrogen–phosphorus detection (Carlo Erba NPD 40 detector). The sensitivity limit reached, even though higher than that obtained with MS detection, nevertheless made it possible to determine urethane in samples and to obtain comparable results with typical chromatographic profiles such as those depicted in Fig. 6.

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