Thermolysis of Carbonates 1–7. A 10–50-mg sample of each of the carbonates was thermolyzed in a glass tube at 250 °C for 10 min. The residue was cooled to room temperature and extracted with methylene chloride. The extract was diluted to 1 mL with methylene chloride in a volumetric flask. Injection of a 10-μL sample into a Varian 3700 gas chromatograph equipped with a 6 ft, 0.125-in. o.d., OV-101 column, was used to examine the extract. An external standard solution of the particular alcohol or phenol component, in conjunction with a Hewlett-Packard 3390A integrater, was used to quantitate the yield of the released component in each case.

Thermolysis GC/MS Analysis. A 25–75-μg sample of the carbonate was weighed into a clean ceramic boat and placed into the section of a quartz tube maintained at room temperature. The remainder of the tube passed through a furnace and was connected to the injection port of a gas chromatograph (Bendix Model 2200). After air from the tube was purged with helium, the sample and boat were pushed into the thermolysis zone maintained at 300 °C. The volatile material was condensed on a 30-m DB-5 fused silica capillary column. After the oven temperature was held at 0 °C for 4 min, the column temperature was heated at 5 °C/min to 280 °C. The chromatographic effluent led directly into the ion source of a Finnigan Model 3300 mass spectrometer. The products were identified from their mass spectral fragmentation patterns.

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Registry No. 1, 94192-23-1; 2, 94192-24-2; 3, 94219-44-0; 4, 94192-22-0; 5, 103478-49-5; 6, 103478-50-8; 7, 103478-51-9; 8, 103478-52-0; 9, 103478-53-1; C_6H_5OCOCl, 1885-14-9; (*Z*)-$ClCO_2(CH_2)_2CH{=}CHCH_2CH_3$, 94192-19-5; 2-$ClCO_2C_6H_4OCH_3$, 2293-75-6; (*Z*)-$HO(CH_2)_2CH{=}CHCH_2CH_3$, 928-96-1; 2-$CH_3OC_6H_4OH$, 90-05-1; glycolaldehyde, 141-46-8; 2-methoxy-4-methylphenol, 93-51-6; 2-isopropyl-5-methylcyclohexanol, 1490-04-6.

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Identification and Quantitation of 1,2,3,4-Tetrahydro-β-carboline-3-carboxylic Acid and 1-Methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic Acid in Beer and Wine

Talmage R. Bosin,* Stephanie Krogh, and Dale Mais

Tetrahydro-β-carboline-3-carboxylic acid (THBC-3-COOH) and 1-methyltetrahydro-β-carboline-3-carboxylic acid (1-MeTHBC-3-COOH) were identified and quantitated in beer and wine. The analytical procedure employed aqueous derivatization with methyl chloroformate to facilitate the isolation, to improve the liquid chromatographic separation, and to eliminate the potential for artifactual formation of these compounds during sample preparation. Identification and quantitation were accomplished through a combination of high-performance liquid chromatography and mass spectrometry. The concentration of THBC-3-COOH in beer and wine ranged between 2.7–10.9 and 0.8–1.7 μg/mL, respectively, and the concentration of 1-MeTHBC-3-COOH ranged between 0.3–4.2 and 1.3–9.1 μg/mL, respectively.

More than a decade ago, it was suggested that some of the effects of ethanol consumption might be mediated by the formation of endogeneous alkaloids formed by the reaction of acetaldehyde with a biogenic amine (Davis and Walsh, 1970; Cohen and Collins, 1970). One example of this process is the reaction of an aldehyde with an (aminoethyl)indole to produce, via a Pictet–Spengler reaction, a 1,2,3,4-tetrahydro-β-carboline (THBC). Such reactions occur readily under physiological conditions (Whaley and Govindachari, 1951), and a generalized reaction is shown in Figure 1.

THBC compounds have been increasingly implicated in alcoholism through their ability to function as neurotransmitters or neutromodulators that alter serotonergic function in the central nervous system (Buckholtz, 1980; Bloom et al., 1982). Acute and chronic administration of THBC compounds to rats has been reported to significantly alter their consumption of ethanol (Geller and Purdy, 1975; Myers and Melchoir, 1977; Tuomisto et al., 1982). Recently several THBC compounds have been identified and quantitated in food and alcoholic beverages

Pharmacology Section, Medical Sciences Program, Indiana University School of Medicine, Bloomington, Indiana 47405.

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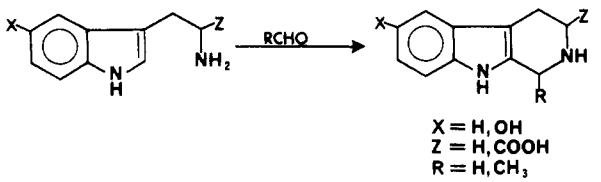
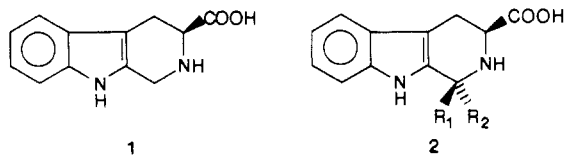


Figure 1. Formation of 1,2,3,4-tetrahydro-β-carbolines by the Pictet–Spengler reaction of an indoleamine with an aldehyde.

(Beck and Holmstedt, 1981; Beck et al., 1983; Bosin et al., 1983). In light of the pharmacological activity of THBC compounds and their potential role in alcoholism, we undertook the analysis of alcoholic beverages for the presence of THBC compounds formed by the reaction of tryptophan with formaldehyde or acetaldehyde to produce 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (THBC-3-COOH, **1**) and 1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (1-MeTHBC-3-COOH, **2**), respectively.



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EXPERIMENTAL SECTION

Materials. L-Tryptophan and 5-methoxy-D,L-tryptophan were purchased from Sigma Chemical Co. (St. Louis, MO), glyoxylic acid and methyl chloroformate were obtained from Aldrich Chemical Co. (Milwaukee, WI), and glass-distilled dichloromethane and methanol were purchased from Burdick and Jackson Labs (Muskegon, MI). All other chemicals were of analytical purity.

THBC-3-COOH (**1**) was synthesized both from L-tryptophan and formaldehyde (Jacobs and Craig, 1936) and from L-tryptophan and glyoxylic acid by the procedure of Vejdelek et al. (1961). 1-MeTHBC-3-COOH (**2**) was prepared by the procedure of Brossi et al. (1973), which gives a 10:1 diastereoisomeric mixture of (−)-(1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**2a**) and (−)-(1*R*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**2b**) of known absolute configuration (Yamada and Akimoto, 1969). The internal standard, 6-methoxy-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (6-OMeTHBC-3-COOH) was prepared from 5-methoxy-D,L-tryptophan and glyoxylic acid as described by Vejdelek et al. (1961).

Sample Preparation. A detailed presentation of the analytical method has been published (Bosin and Jarvis, 1985), and an abbreviated form appears below.

A 1.0-mL aliquot of each alcoholic beverage was added to 125 × 15 mm glass tubes containing 60 μL (6 μg) of 6-OMeTHBC-3-COOH. The sample was treated with 1.0 mL of 1.0 M dipotassium hydrogen phosphate solution (pH 7.2) and 50 μL of methyl chloroformate, vortexed, and allowed to stand for 5 min. The sample pH was increased to pH 9.5 by the addition of 0.50 mL of a saturated sodium carbonate solution (pH 11.5), 50 μL of methyl chloroformate was again added, and the sample was vortexed and allowed to stand for 10 min. Extraction of the sample with 6.0 mL of dichloromethane removed derivatized basic precursors and basic THBC compounds. The remaining aqueous phase was carefully acidified with concentrated hydrochloric acid to a final pH of 3 and extracted with 6.0 mL of dichloromethane. Evaporation of the dichloromethane under nitrogen yielded the compounds of interest, which were stable for more than 7 days at −80 °C. Blank samples carried through the analytical procedure did not yield any compound **1** or **2**. Control samples containing L-tryptophan (100 μg) or alcoholic beverage samples worked up in the presence or absence of semicarbazide (1 mg) did not produce any artifactual **1** or **2**. Samples were dissolved in methanol/0.01 M sodium acetate (pH 4.6) (45:55) prior to chromatographic analysis.

Liquid chromatography was performed on a Varian 5020 liquid chromatograph (Várian, Palo Alto, CA) equipped with a universal loop injector, a 5-cm column guard packed with Vydac reversed-phase hydrocarbon (Separations Groups, Hesperia, CA), and a 5-μm Zorbax ODS, 25 cm × 4.6 mm i.d. column (DuPont, Wilmington, DE). Samples were eluted at a flow rate of 0.8 mL/min with a solvent system composed of methanol/0.01 M sodium acetate (pH 4.6) (45:55). Fluorescence detection was achieved on a Fluorichrom detector (Varian, Palo Alto, CA) equipped with a deuterium arc source, a 200I excitation filter, and a Corning 7-60 band filter (360 mm) for emission. Chromatograms were recorded, and data were collected on a Hewlett-Packard Model 3390 A reporting integrator (Hewlett-Packard, Avondale, PA) operating in the peak height mode.

Mass spectra were recorded with a direct probe and an LKB 9000 mass spectrometer equipped with a Teknivent IBM PC data system (Technivent Corp., St. Louis, MO). The mass spectrometer conditions were as follows: electron energy 70 eV; trap current 60 μA; accelerator voltage 3.5 kV.

Quantitation. Calibration curves were constructed by plotting the peak height ratios (THBC-3-COOH/6-OMeTHBC-3-COOH and 1-MeTHBC-3-COOH/6-OMeTHBC-3-COOH) of standard samples against the concentrations of THBC-3-COOH and 1-MeTHBC-3-COOH, respectively. The standard samples were prepared by adding differing amounts of THBC-3-COOH (0.50–10.0 μg) and 1-MeTHBC-3-COOH (0.50–10.0 μg) to 125 × 15 mm glass tubes containing 1.0 mL of water, 60 μL (6 μg) of 6-OMeTHBC-3-COOH, and 50 μL (1 mg) of semicarbazide. All standard samples were carried through the workup procedure (vide supra). The concentrations of THBC-3-COOH and 1-MeTHBC-3-COOH were determined from the peak height ratios of each sample by reference to the calibration curve.

RESULTS AND DISCUSSION

The analytical procedure used in this study has been described in detail (Bosin and Jarvis, 1985). The procedure is based on the aqueous derivatization of THBC compounds with methyl chloroformate and has been shown to facilitate the isolation of these compounds from an aqueous medium, to eliminate the potential for the artifactual formation of THBC compounds during sample preparation, and to markedly improve their high-performance liquid chromatographic (HPLC) separation. When this method was applied to aqueous samples of **1** and **2**, the derivatization reaction was found to be quantitative and their recovery in the extraction step was found to be >90% by comparing the peak heights of extracted and nonextracted samples. The limit of sensitivity (4 times background) of this method for **1** and **2** was less than 10 ng/sample, while the reproducibility of the method in the analysis of the same beer and wine samples (n = 4) gave coefficients of variation of 3.1–4.5%.

The identification of **1** and **2** in the alcoholic beverage samples was based on a comparison of their reversed-phase HPLC retention times to the retention times of authentic compounds and a comparison of the mass spectra obtained by collecting and evaporating the corresponding HPLC peaks to the spectra produced by synthetic **1** and **2**. A

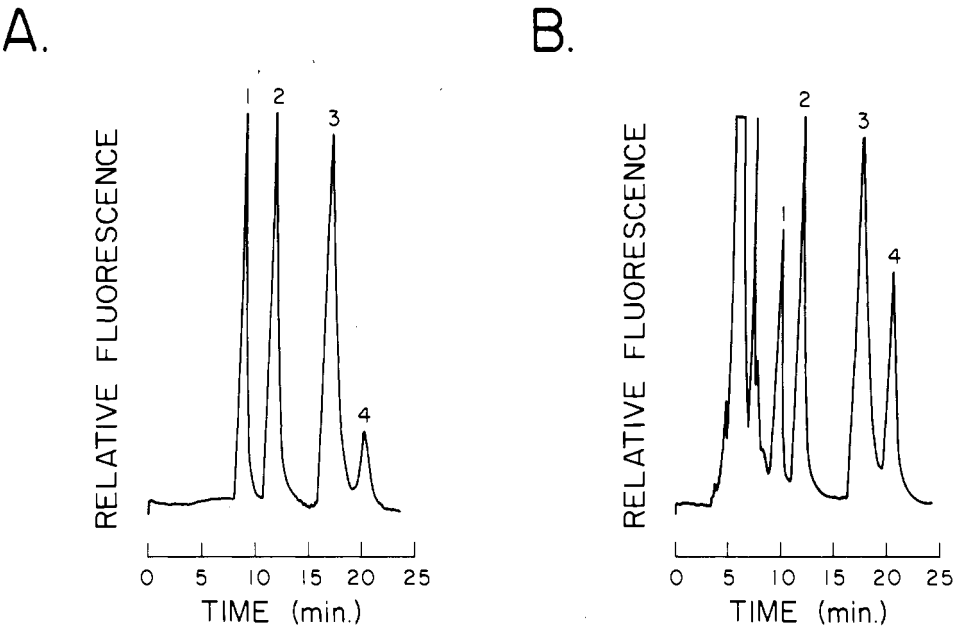
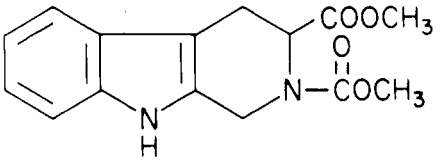


Figure 2. High-performance liquid chromatograms of derivatized THBC-3-COOH (1), 6-OMeTHBC-3-COOH (2), (1*S*,3*S*)-1-MeTHBC-3-COOH (3), and (1*R*,3*S*)-1-MeTHBC-3-COOH (4) in authentic (A) and beer (B) samples.



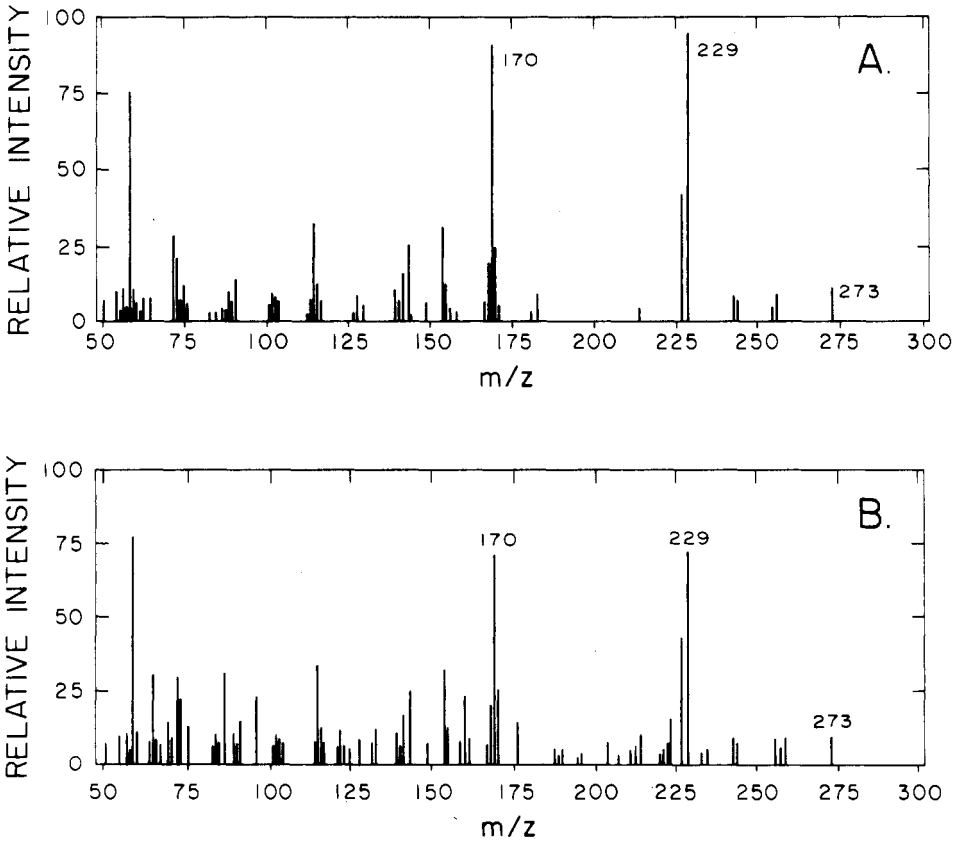


Figure 3. Electron impact mass spectra of authentic derivatized THBC-3-COOH (A) and THBC-3-COOH isolated from beer (B).

typical chromatogram of authentic **1** and **2** and the internal standard 6-OMeTHBC-3-COOH is presented in Figure 2A and demonstrates the facile separation obtained with these derivatized samples. Synthetically prepared **2** has been shown (Brossi et al., 1973) to exist as a 10:1 diastereoisomeric mixture of **2a** to **2b** of known absolute configuration (Yamada and Akimoto, 1969), and these diastereomers are readily separated by this procedure. Comparison of the

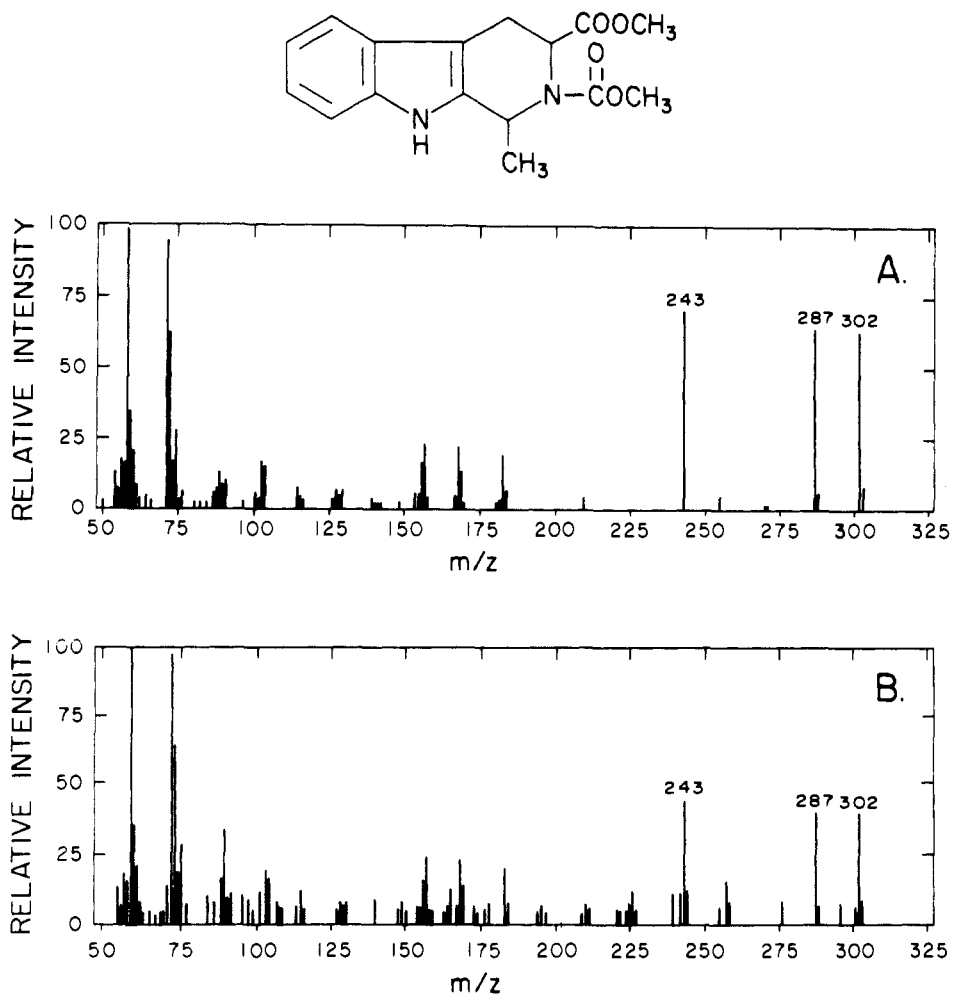


Figure 4. Electroon impact mass spectra of authentic derivatized 1-MeTHBC-3-COOH (A) and 1-MeTHBC-3-COOH isolated from beer (B).

chromatogram presented in Figure 2A to the chromatogram obtained from the analysis of a beer sample (Figure 2B) indicates the presence of compounds possessing retention times identical with those of **1** and **2**. Further confirmation was provided by adding derivatized authentic **1** and **2** to the sample and observing a potentiation of the heights of peaks 1, 3, and 4.

The complete confirmation of the identity of these peaks was achieved by repetitively collecting and evaporating the HPLC effluent corresponding to peaks 1, 3, and 4 in beer and wine and obtaining a complete mass spectrum of each. The mass spectrum of derivatized **1** is shown in Figure 3A and was obtained on the methyl ester that was prepared by diazomethane treatment. The identified ions were m/z 273 ($M^{\cdot+} - CH_3$), 229 ($M^{\cdot+} - CO_2CH_3$), and 170 ($M^{\cdot+} - CO_2CH_3$, CO_2CH_3). A molecular ion (m/z 288) could be observed with a lower electron energy. Comparison of the mass spectrum shown in Figure 3A with that in Figure 3B, which was obtained by collecting peak 1 in the HPLC chromatogram (Figure 2B), indicated no significant differences in the major ions or their intensity ratios. Similarly, when the HPLC effluent corresponding to peaks 3 and 4 in Figure 2B were collected, evaporated to dryness, and treated with diazomethane, mass spectra were obtained (Figure 4B) that contained the same ions and intensity ratios as those found in authentic **2** (Figure 4A). The identified ions in Figure 4 corresponded to m/z 302 ($M^{\cdot+}$), 287 ($M^{\cdot+} - CH_3$), and 243 ($M^{\cdot} - CO_2CH_3$). Thus, the identity of these compounds in beer and wine was established on the basis of the identical HPLC and mass spectral properties when compared to standard compounds.

Table I. Concentrations of THBC-3-COOH and 1-MeTHBC-3-COOH in Beer

beer	concn, μg/mL	
	THBC-3-COOH	1-MeTHBC-3-COOH
Budweiser	4.16	3.63
Bud Light	2.06	0.32
Stroh	3.39	0.96
Stroh Light	3.65	1.12
Miller	3.58	1.15
Lite	2.72	0.45
Coors	7.06	2.14
Old Style	7.40	4.24
Olympia	3.18	0.55
Pabst	10.86	2.16

The quantitation of compounds **1** and **2** in the various samples was accomplished by determining the peak height ratios of each compound relative to the internal standard (peak 2, Figure 2) and reference to a calibration curve. The quantitative data obtained from the analysis of select beer samples are presented in Table I, and the corresponding data obtained with various wine samples appear in Table II. In both the beer and wine samples the diasteroisomeric mixture ratios of **2a** to **2b** was approximately 2:1, in contrast to the 10:1 ratio found in synthetically prepared **2** (Brossi et al., 1973). For quantitative purposes the diastereomers were considered as a single compound.

The data found in Tables I and II indicate that compound **1** is present in much higher concentrations in beer

Table II. Concentration of THBC-3-COOH and 1-MeTHBC-3-COOH in Wine

wine	concn, μg/mL	
	THBC-3-COOH	1-MeTHBC-3-COOH
Ingelnook Rhine	1.44	9.10
Sebastiani Rhine	1.32	8.32
Sebastiani Chalblis	1.09	5.03
Gallo Chenin Blanc	ND	1.30
Sebastiani Burgundy	0.81	3.72
Lawrence Zinfandel	1.70	6.27
Cribari Chianti	1.53	7.44

than compound 2, while the opposite is true in the wine samples; i.e., compound 2 is present in much higher concentration than 1. Previous work has shown a definite relationship between the ethanol content of the beer and the concentration of 6-OH-1-MeTHBC (Beck al., 1983); however, in the present study there does not appear to be a clear relationship between the concentration of 1 and/or 2 in beer and the ethanol content. In addition, no relationship was observed between the type of wine and the amounts of 1 and 2 found.

The origin of 1 and 2 in beer and wine is unknown and most likely is the result of both a Pictet–Spengler reaction between tryptophan and formaldehyde or acetaldehyde and their natural presence in the different brewing ingredients or grape sources. Of greater potential importance is the markedly higher levels of 1 and 2 (μg/mL) in comparison to those previously reported (ng/mL) for 1-MeTHBC (Beck and Holmstedt, 1981), 6-OHTHBC (Bosin et al., 1983), and 6-OH-1-MeTHBC (Beck et al., 1983). Since THBC compounds exhibit significant pharmacological activities (Buckholtz, 1980; Bloom et al., 1982), the presence of such high concentrations of 1 and 2 in these beverages suggests the potential for behaviorial and toxicological effects in both the normal and the alcoholic individual. The pharmacological and toxicological potential of 1 and 2 needs to be fully defined.

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Volatile Constituents of Greek Ouzo

Michael G. Kontominas[1]

Ouzo is the most popular distilled spirit consumed in Greece. Its characteristic aroma and flavor are attributed to anethole, the main constituent of anise seed. Volatile components of two different ouzo products, Sans Rival and Callicounis, were extracted with Freon 11, centrifuged, concentrated, and analyzed by GC and GC/mass spectrometric techniques. A total of 26 components was identified. Differences in aroma and flavor between the two ouzo products are attributed to benzaldehyde and other minor components.

INTRODUCTION

Many chemical compounds contribute to the organoleptic character of distilled spirits (Eriksson, 1978). Fatty acids, fatty acid esters, carbonyl, phenolic, sulfur, and nitrogen compounds as well as lactones, acetals, hydrocarbons, etc., have been reported to be responsible for the characteristic aroma and flavor of such spirits (Postel and Adam, 1961; Drawert and Rapp, 1965; Drawert et al., 1967; Nykänen et al., 1968; Kahn, 1969; Reinhard, 1971; Nykänen, 1972; Salo, 1973; Ronkainen et al., 1973; Nosko, 1974; Endres et al., 1976; Postel and Adam, 1976, 1977; Reinhard, 1977; Postel and Adam, 1979, 1980, 1984; Yavas and Rapp, 1985). Ouzo, the most popular distilled spirit consumed in Greece, in manufactured from the "rachis", the stem structure of the grape cluster, which are distilled.

Flavorants are then added to the distillate, the most common of which is anise seed oil. The main component comprising 80–90% of anise seed oil is anethole or 1-methoxy-4-(1-propenyl)benzene (Guenther, 1950). Other compounds identified in anise oil include (*p*-methoxyphenyl)acetone (anethole isomer), β-pinene, camphene, *d*-fenchane, dipentene, acetaldehyde, α-phellandrene, etc.

The economical importance of ouzo as both a domestic and an export product in conjunction with the fact that

Department of Food Science, Cook College, Rutgers University, New Brunswick, New Jersey 08903.
[1]Present address: Department of Food Chemistry, University of Ioannina, Ioannina, Greece.

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