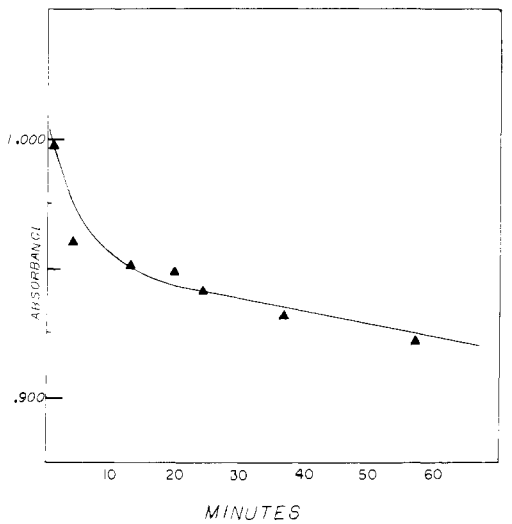

Figure 1. Blank reaction curve showing the nature of the reaction.

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LITERATURE CITED

Amerine, M., "Laboratory Procedures for Enologists", AAS, Davis, Calif., 1965.
Bergmeyer, H., "Methods of Enzymatic Analysis", AP, New York, N.Y., 1963.
Bergmeyer, H., "Methods of Enzymatic Analysis", Vol. 1, AP, New York, N.Y., 1974a.
Bergmeyer, H., "Methods of Enzymatic Analysis", Vol. 3, AP, New York, N.Y., 1974b.
Boyer, P. D., *Enzymes*, 2nd Ed., **6**, 95 (1962).
Daubt, C. E., Ough, C. S., *Am. J. Enol. Viticult.* **24**, 125 (1973).
Holz, G., Bergmeyer, H. U., in H. Bergmeyer., "Methoden der Enzymatishen Analyse", 2nd ed, Vol. 2, Verlag Chemie Weinhiemer, 1970, p1486.
Kaplan, N. O., *Enzymes, 2nd Ed.*, **3**, 105 (1962).
Lundquist, F., Fugmann, U., Rassmussen, H., *Biochem. J.* **80**, 393 (1961).
McCloskey, L. P., Abstract of paper presented at the Annual Meeting of the American Society of Enologists, 1975.
Meyers, L. E., MacDonald, R. H., Eskelson, C. D., personal communications, 1974.
Postel, W., Drawert, F., MacCagnan, G., *Chem. Mikrobiol. Technol. Lebensm.* 1, 11–14 (1971).
Rose, I. A., *Methods Enzymol.* **2**, 591 (1955).
Rose, I. A., *Enzymes, 2nd Ed.*, **6**, 115 (1962).

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Spectrofluorometric Determination of β-Asarone in Sweet and Dry Vermouths

Edward J. Wojtowicz

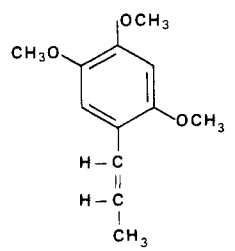
A spectrofluorometric method for the quantitative determination of β-asarone in sweet and dry vermouths has been developed. After steam distillation of the β-asarone from the vermouth, the compound is partitioned into hexane, which is then washed with 1 N NaOH, 1 N HCl, and water. The β-asarone is then determined fluorometrically. The presence of β-asarone is confirmed by thin-layer chromatography of the same solution (0.5 μg detectability). The procedure gives greater than 90% recovery of 1 ppm of β-asarone in both sweet and dry vermouths.

Oil of calamus, derived from the dried rhizome of *Acorus calamus* Linn., is used as a minor flavoring agent at levels below 5 ppm and up to 10–30 ppm in bitters, liqueurs, and vermouths (Miller, 1973). Studies (Gross et al., 1967; Taylor et al., 1967) have shown that malignant intestinal tumors form in rats fed a diet containing up to 5000 ppm of oil of calamus. Use of the root, oil, or extract in food has been banned in the United States (Code of Federal Regulations, 1974a); however, it is still used in certain European countries (Hall, 1973).

The β-asarone content of the oil derived from the tetraploid variety of Indian *Acorus calamus* Linn. has been reported to be 82% (Guenther, 1952) while oil from the European variety contains about 5% β-asarone (Larry, 1973).

Column chromatography (Chopra et al., 1965) and gas–liquid chromatography (Larry, 1973; Usseglio-Tomasset, 1966) have been used for the quantitative determination of β-asarone, while thin-layer chromatography (TLC) (Stahl, 1965) has been used for the qualitative identification of the components in calamus.

These methods lack sensitivity in the fractional part per million range which is required when dealing with a carcinogenic compound. β-Asarone is the cis isomer of 2,4,5-trimethoxy-1-propenylbenzene, and lends itself well to fluorescence analysis. A search of the literature showed no previous reference to the use of fluorescence for β-asarone analysis.



A method has been developed that utilizes spectrofluorometry for quantitative estimation of β-asarone in sweet and in dry vermouths and TLC for qualitative identification.

Food and Drug Administration, 599 Delaware Ave., Buffalo, New York 14202.

EXPERIMENTAL SECTION

Apparatus and Materials. The steam distillation apparatus described in "Official Methods of Analysis" (1975) was used, except that a 500-ml, short-necked flask heated with an electric heating mantle was substituted for the distillation tube and burner.

A 500-ml Kuderna-Danish evaporative concentrator with 24/40 column connection and 19/22 lower joint with a suitable 10-ml concentrator tube and Snyder-type distilling column (Kontes Glass Co.) was used.

β-Asarone standard was supplied by the Division of Toxicology, Food and Drug Administration, Washington, D.C.

Caution. Special precautions are necessary when handling carcinogenic compounds. These precautions are outlined in the Code of Federal Regulations (1974b).

Procedure. A 50-ml portion of sweet or dry vermouth was transferred to a 500-ml distillation flask containing 40 ml of water and 10 ml of saturated NaCl solution. The distilling tube was inserted into the flask and connected to the water condenser. The water in the steam generator was brought to a boil while the sample was heated and 150 ml of distillate was collected. The distillate was transferred to a 250-ml separatory funnel, cooled, and extracted with 50.0 ml of spectral grade hexane (Burdick and Jackson Laboratories, Inc.). After settling, the aqueous layer was discarded and the hexane was serially washed with 20 ml of 1 N NaOH, 20 ml of 1 N HCl, and 20 ml of water. There was no apparent loss of hexane into the aqueous layer in the washing steps.

Fluorometric Determination. A portion of the hexane layer was transferred directly to a 1-cm fluorometric cell and the fluorescence was measured, using a Perkin-Elmer MPF-3 recording spectrofluorometer with excitation at 310 nm and emission at 350 nm. A slit width of 7 nm for excitation and emission and a sensitivity setting of 0.3 were used, with hexane as a blank.

The fluorescence of standard solutions of β-asarone was linearly dependent upon concentration from 0 to 2 μg/ml of hexane.

Thin-Layer Chromatography. Eastman Chromagram sheets of silica gel and alumina, each with a fluorescent indicator, were used. The hexane extract was dried through Na_2SO_4, evaporated to about 5 ml in the Kuderna-Danish evaporator equipped with a 10-ml concentrator tube and Snyder-type distilling column, and further evaporated to 1 ml with air. Standards, samples, and fortified samples were chromatographed, using benzene as the development solvent. Equivalents of 0.5 ml of sample were spotted with 0.5 μg of standard material. Spots could easily be detected at this level under ultraviolet light at 254 nm. R_f values of 0.20 on silica gel and 0.30 on alumina were observed for β-asarone. Development time in a saturated chamber was about 0.5 h for 10 cm.

When the silica gel sheets and an ethanol–acetic acid–chloroform (10:2:88) solvent system were used, an R_f of 0.50 was observed for β-asarone.

RESULTS AND DISCUSSION

β-Asarone was found to have two excitation wavelengths in hexane at 264 and 310 nm. Both excitation wavelengths gave an emission at 350 nm (Figure 1). The 310-nm excitation wavelength was selected due to an approximate threefold increase in intensity of the 350-nm emission over that of the 264-nm excitation. In addition, background interferences at the 350-nm emission were minimized by excitation at 310 nm (Figures 2 and 3). The slit width of 7 nm was chosen to give adequate resolution between excitation and emission peaks.

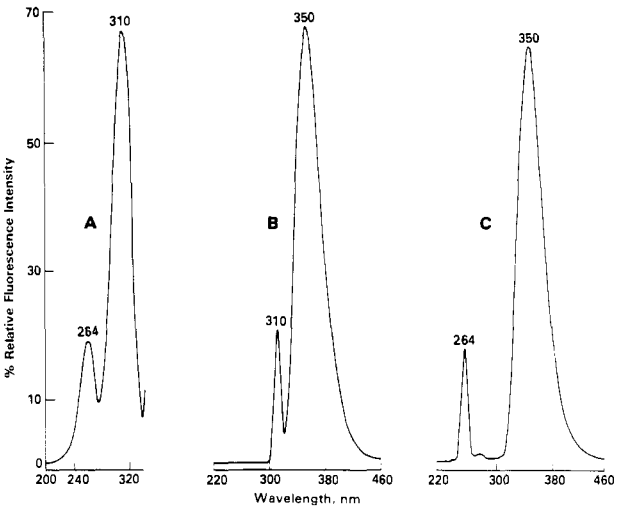


Figure 1. Uncorrected fluorescence spectra of β-asarone standard (1 μg/ml of hexane): (A) excitation spectrum with emission monochromator set at 310 nm, sensitivity setting 0.3; (B) emission spectrum with excitation monochromator set at 310 nm, sensitivity setting 0.3; (C) emission spectrum with excitation monochromator set at 264 nm, sensitivity setting 1.0.

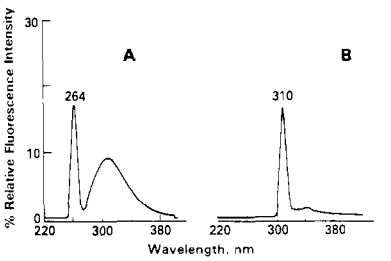


Figure 2. Uncorrected fluorescence spectra of β-asarone-free vermouth: (A) excitation 264 nm, sensitivity setting 1.0; (B) excitation 310 nm, sensitivity setting 0.3. Note interference in sample A.

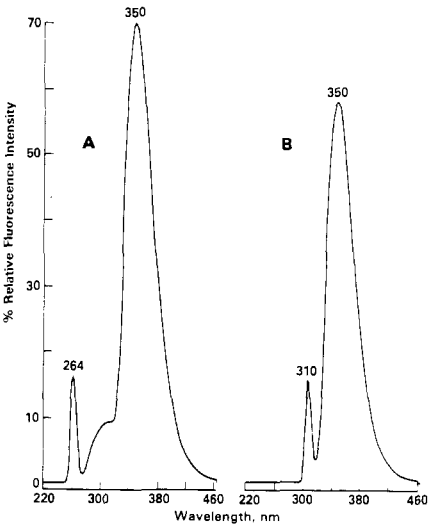


Figure 3. Uncorrected fluorescence spectra of vermouth fortified at 1 ppm with β-asarone standard: (A) excitation 264 nm, sensitivity setting 1.0; (B) excitation 310 nm, sensitivity setting 0.3. Note interference in sample A.

Four samples each of different brands of imported sweet and dry vermouths were analyzed according to the procedure described herein, and no β-asarone was detected. The fluorometer was preset to give 70–80% relative fluorescence intensity with a 1-ppm standard β-asarone

solution so that levels down to the 0.1–0.2-ppm range could be detected. Recoveries were performed using five 50-ml portions of sweet and dry vermouths fortified at 1 ppm with β-asarone. The average recovery of β-asarone was 96.3 ± 0.9% from the sweet vermouth and 93.0 ± 0.9% from the dry vermouth.

The entire procedure including distillation, shakeout, spectroscopy, evaporation, and TLC of a sample extract can easily be done in about 2 h. The method presented here offers detection at 1 ppm for β-asarone in sweet and dry vermouths with ±1% precision and recoveries of better than 90%.

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LITERATURE CITED

Chopra, M. M., Vashist, U. N., Handa, K. L., *J. Chromatogr.* **17**, 195 (1965).
Code of Federal Regulations, Title 21, 3.65a,b,c, 1974a.
Code of Federal Regulations, Title 29, 1910.93c, 1974b.
Gross, M. A., Jones, W. I., Cook, E. L., Boone, C. C., *Proc. Am. Assoc. Cancer Res.* **8**, 24, Abstract 93 (1967).
Guenther, E., "The Essential Oils", Vol. VI, Van Nostrand Co., New York, N.Y., 1952, p 115.
Hall, R. L., "Toxicants Occurring Naturally in Foods", 2nd ed, National Academy of Sciences, Washington, D.C., 1973, Chapter 20, p 453.
Larry, D., *J. Assoc. Off. Anal. Chem.* **56**, 1281 (1973).
Miller, J. A., "Toxicants Occurring Naturally in Foods", 2nd ed, National Academy of Sciences, Washington, D.C., 1973, Chapter 23, p 530.
"Official Methods of Analysis," 12th ed, Association of Official Analytical Chemists, Washington, D.C., 1975, p 365, 20.093.
Stahl, E., Ed., "Thin Layer Chromatography", Academic Press, New York, N.Y., 1965, pp 197–205.
Taylor, J. M., Jones, W. I., Hagan, E. C., Gross, M. A., Davis, D. A., Cook, E. L., *Toxicol. Appl. Pharmacol.* **10**, 405, Abstract 72 (1967).
Usseglio-Tomasset, L., *Estratta Ind. Agric.* **4**, 3 (1966).

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Measurement of *N*-Carbethoxyproline and *N*-Carbethoxyglycine in Model Solutions and in Wine

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The *N*-carbethoxy derivatives of proline and glycine were made and identified. The formation of these derivatives in a wine medium as a result of the addition of diethyl pyrocarbonate (DEPC) was demonstrated. A simple method of extraction, concentration, methylation, reextraction, and determination on a gas–liquid chromatography/Coulson system was developed. The formation of *N*-carbethoxyproline in wine, treated with DEPC at 100 mg/l., ranged from 0.3 to 5.2 mg/l. for seven samples. The amount of *N*-carbethoxy derivative formed in the model solutions and in wines is proportional to the amount of amino acid present and the amount of DEPC added. As pH increases in the pH range of wine, the amount of carbethoxyproline produced also increases.

Boehm and Mehta (1938) first described the ability of diethyl pyrocarbonate (DEPC) to react with amines to form *N*-carbethoxy derivatives. Further, Thoma and Rinke (1959) elucidated this reaction with amines. Thoukis et al. (1962) treated DEPC with a slurry of glycine and formed a crystalline solid with a melting point of 68–72 °C. It was resistant to hydrolysis in boiling 6 N HCl. They also formed a similar product with proline. Rosanati (1964) synthesized several *N*-carbethoxy amino acids by refluxing the amino acid with DEPC in absolute ethanol. Larrouquère (1964) showed that DEPC reacted also with primary amines and amino acids but said they did not react below pH 6.0. Duhm et al. (1966) used ^{14}C-labeled DEPC to investigate possible reactions with 41 common beverage constituents at pH 3–4. They found about 0.5% residual activity when amino acids were tested.

Mülhrad et al. (1967) examined at alkaline pH the reaction products between DEPC and the amino acids glycine, histidine, tyrosine, arginine, cysteine, and tryptophan. From electrometric titration and infrared spectra they concluded that the amino groups were carbethoxylated, as were also the N-1 of the imidazole ring of histidine, the guanido group of arginine, the phenolic hydroxyl of tyrosine, and the sulfhydryl of cysteine. At acid pH, only the N-1 of the imidazole was reactive. Proline is the main amino acid remaining in wine after fermentation (Ough, 1968; Ough and Stashak, 1974).

This work was done to verify the formation of *N*-carbethoxy derivatives in the reaction between DEPC and the amino acids glycine and proline at pH 3–4, to define the parameters of formation by use of model solutions, and to develop a method of quantification of these compounds in wine.

METHODS AND MATERIALS

The pure carbethoxy derivatives were prepared in a manner similar to that of Carter et al. (1955). The original extracted product was an oil phase which was dissolved in 500 ml of hot petroleum ether and put into a deep freeze overnight. Decanting off the supernatant and recrystallizing twice more in the same manner produced a good crystalline product.

The melting points were determined in capillary tubes in slowly heated mineral oil.

In preparation for determinations of the infrared spectra, the two carbethoxy compounds were mixed, 1.0 or 1.5 mg, with 100 mg of KBr (Aldrich Chemical Co., infrared grade) and pelletized, or dissolved in a small portion of methylene chloride and put onto salt crystal. A Beckman 5A infrared

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