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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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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spectrophotometer was used.

As a further check on purity, the samples were spotted on 300 μm thick silica gel G plates (20 × 20 cm) and developed in 70% 1-butanol and 30% ammonia. The plates were dried and sprayed with $KMnO_4$ in H_2SO_4.

Used for positive identification of the proline derivative by gas–liquid chromatography (GLC)/mass spectra was a Hewlett Packard 5930A GC–MS with a Systems Industries 150 data system. Chromatography was done with a 6 ft × 2 mm glass column, 3% OV-17 on Chromosorb W (H.P.) with the temperature programmed from 80 to 230 °C at 16 °C/min. The ionization energy was 70 eV, and the mass range scan was generally 15 to 215 amu.

Model solutions were prepared to determine the effect of DEPC and amino acid concentrations on the amount of carbethoxy compounds formed. Eleven percent v/v ethanol–water, 0.2 M K_2HPO_4 in 11% v/v ethanol, and 0.1 M citric acid in 11% v/v ethanol in desired proportions were mixed to get pH solutions of 3.00, 3.25, 3.50, 3.75, and 4.00. The ethanol–water 11% v/v was used as 75% of the mixture each time, and the other two components were varied to get the desired pH. Varying amounts of DEPC (Bayer) and amino acids were added.

After 2 days at 20 °C the samples were extracted three times, once with a 200-ml portion of ethyl acetate and then twice with 100-ml portions. The extracts were dried by passing through anhydrous sodium sulfate and then concentrated with a Kuderna-Danish evaporator with a three-ball Snyder column on a steam bath. When the sample was concentrated to less than 10 ml it was transferred, with proper ethyl acetate rinsing of the Kuderna-Danish evaporator, into a Kontes micro concentrator tube with a three-ring reflux column and concentrated to a few milliliters. It was cooled and rinsed into a 10-ml round-bottomed flask fitted with a reflux condenser. The method of Brenner and Huber (1953) was used to methylate the carboxyl group. The mixture should contain about 10 μg of *N*-carbethoxy amino acid ester. The sample was diluted with 5 ml of water saturated with NaCl and extracted with 1 ml of ethyl acetate for GLC analysis. External standards were prepared each time, extracting and deriving in the same manner as above.

Additional model-wine-solution tests made were to determine the effects of amino acid and DEPC concentration on the amount of product formed.

Quantitative analysis of the samples used a GLC (Varian 1700) connected to a Tracor Coulson nitrogen detector. The column used was 6 ft × 0.25 in. o.d., glass (4 mm i.d.), packed with 3% FFAP (Supelco, Inc.) on Chromosorb GAW support. The usual injection was about 5 μl. The response to injections was linear from 5 to 500 ng. Conditions were as follows: He gas flow, 30 ml/min (GLC); oven temperature, 180 or 200 °C isothermal; injector temperature, 260 °C; Coulson block temperature, 240 °C; pyrolysis oven, 820–840 °C; and, in the nitrogen mode, vent time, 2 min; retention times, respectively, about 7 and 10 min for *N*-carbethoxyglycine (methylated) and *N*-carbethoxyproline (methylated) for 200 °C and 13.8 and 18.0 min at 180 °C. The peak areas were measured with a Hewlett-Packard 9810A computer and a hand-held digitizer, 9864A. An average was taken from three scans of the areas.

An F&M Model 700 GLC with thermal-conductivity detector was used to separate samples for collection in capillary tubes for analysis of infrared spectra.

Wine samples used were prepared by the authors at the University's bonded experimental winery from grapes grown in the vineyards of the University of California or

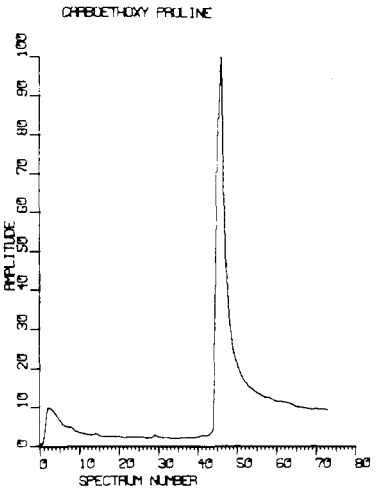

Figure 1. Reconstructed mass chromatogram of *N*-carbethoxyproline.

at supervised field stations. The wines were analyzed for pH (Beckman Research Model) and for proline by the method of Ough (1969). Seven wine samples (250 ml) were treated (in duplicate) with DEPC at 100 mg/l. An untreated control was maintained for each. In addition, a sample (250 ml) of each was fortified with *N*-carbethoxyproline at 1 mg/l. A model wine solution (250 ml) was also fortified with *N*-carbethoxyproline at 1 mg/l. After 72 h at 20 °C a 100-ml portion of each was extracted with 100, 75, and 75 ml of ethyl acetate. The three extracts were combined, dried and concentrated, methylated, reextracted, and measured on the GLC–Coulson system as described. Concentrations were calculated on the recorder response compared with the recorder response of the external standard (*N*-carbethoxyproline added at 1.0 mg/l. to 250 ml of red wine, no DEPC added). The complete procedure of extraction through reextraction was carried out as with treated sample.

RESULTS AND DISCUSSION

Synthesis and Verification. The *N*-carbethoxy synthesis produced a 50% yield of product. The melting point was 71–72 °C for the glycine derivative and 62–63 °C for the proline derivative. The melting point data for the *N*-carbethoxyglycine agree with published values and published infrared (ir) spectra (Mühlrad et al., 1967; Thoukis et al., 1962). No literature verification was available for the melting point or ir spectra for the *N*-carbethoxyproline. Others had failed in attempts to crystallize. The ir spectra of the two forms (crystal and oil) show a primary difference in the size of the 3450-cm^{-1} peak and the presence of 2000- and 1315-cm^{-1} peaks in the crystalline form. The very large peak from 1730 to 1690 cm^{-1} can be attributed to the C=O stretch of the carboxyl combined with the C=O stretch of the amide.

The thin-layer chromatography showed only one compound to be present.

For further positive identification of the *N*-carbethoxyproline a portion was dissolved in ethyl acetate and a mass scan made on a GLC–mass spectrometer. The spectra were recorded at intervals as shown in Figure 1. The printed spectrum (Figure 2) was obtained by subtracting spectrum no. 49 from no. 47 to eliminate background and column bleed.

The small peak at m/e 187 can be assumed to be the molecular ion since it represents the molecular weight of *N*-carbethoxyproline and is separated an appropriate number of mass units from the nearest fragment ion peak. Very little of this ion would be expected because of the

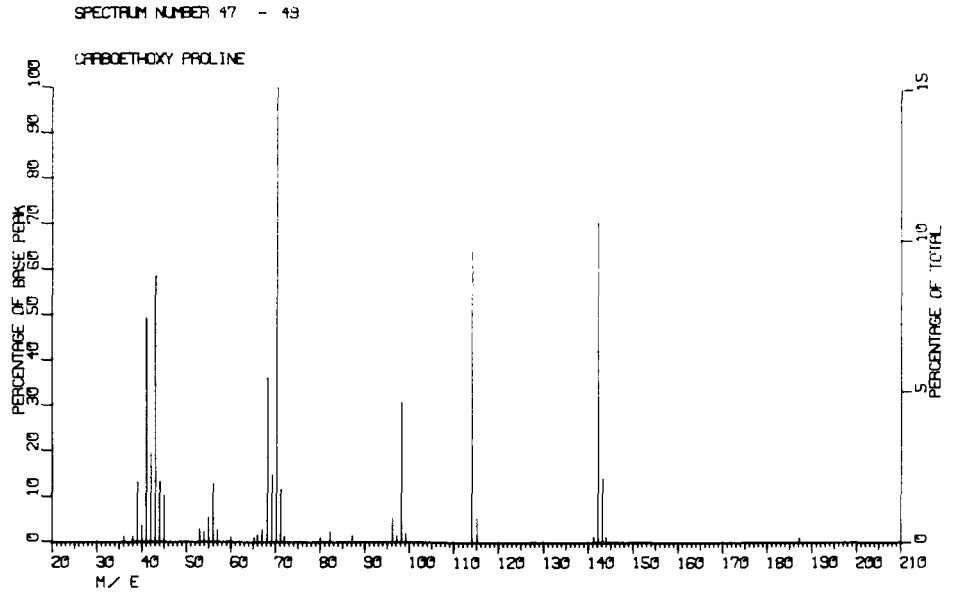

Figure 2. Mass spectrum of *N*-carbethoxyproline (spectrum no. 47–49).

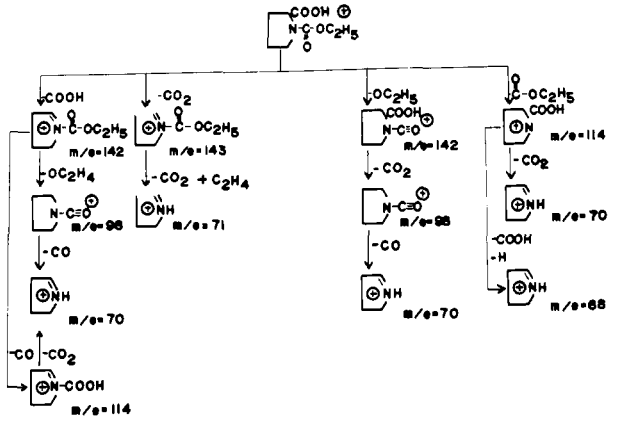

Figure 3. Proposed fragmentation pattern of *N*-carbethoxyproline.

lability of the carboxyl and carbethoxyl functions of the molecule. The major peaks occur at m/e 142, 114, 98, 70, 43, and 41. There are numerous possible fragment schemes. One such (Figure 3) can account for some of the major fragmentation. One might expect the carboxyl to leave entirely; because of the McLafferty rearrangement it would probably be inhibited by the sterically fixed position of the δ-carbon in the pyrrolidine ring. The cluster of fragments at less than m/e 60 is less important but probably includes C_2HN (m/e 39), C_2H_3N (m/e 41), C_2H_5N (m/e 43), and C_2H_5O (m/e 45). The m/e 70 peak is characteristic of compounds such as proline and pyrrolidine and usually represents the stabilized compound shown in Figure 3. The formation of this compound would require some molecular rearrangements during cleavage.

Without more detailed study, including higher resolution and identification of metastable peaks, it cannot be concluded which of these pathways, if any, predominate, although it can be concluded with some certainty that the original product is *N*-carbethoxyproline.

Measurement. The *N*-carbethoxy amino acids are less ionized at lower pH (3.0–4.0) than the corresponding amino acids and can be extracted with organic solvents. The *N*-carbethoxy amino acids can then be methylated, reextracted, separated, and quantified on the GLC–Coulson arrangement.

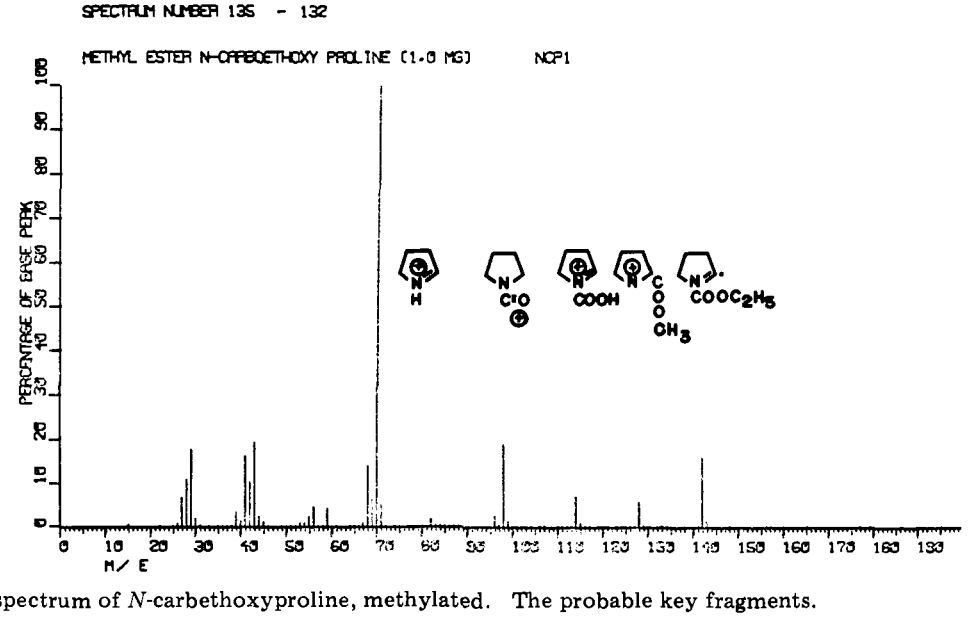

Figure 4. Mass spectrum of *N*-carbethoxyproline, methylated. The probable key fragments.

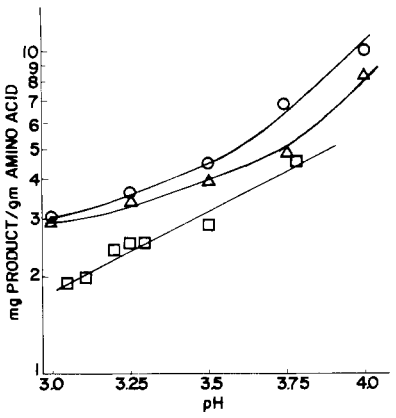

Figure 5. Relationship of milligrams of *N*-carbethoxy products formed per gram of amino acid in model wine and wine to the pH of the system using 100 mg/l. of diethyl pyrocarbonate: (○) *N*-carbethoxyproline in model solution; (△) *N*-carbethoxyglycine in model solutions, and (□) *N*-carbethoxyproline in wine.

Table I. *N*-Carbethoxyproline and *N*-Carbethoxyglycine Produced as a Function of Diethyl Pyrocarbonate Concentration at pH 3.5

DEPC concn, mg/l.	mg of *N*-carbethoxy-glycine per g of Gly	mg of *N*-carbethoxy-proline per g of Pro
0	0	0
50	1.8	2.6
100	3.9	4.0
200	8.1	9.6
400		15.8

The mass spectrum of the methylated *N*-carbethoxyproline is shown in Figure 4, along with some proposed fragment structures. The ir spectrum of the methylated *N*-carbethoxyproline was almost identical with that of the unmethylated product except for a marked sharpening of the peak at 1740 cm^{-1}, caused by the C=O of the ester. This sharpening is expected because of elimination of the resonance structures associated with the carboxyl group. Also indicative of ester formation is the absence of the peak at 3400 cm^{-1}, caused by the –H stretch of the hydroxyl portion of the carboxyl group. This and the fragments of the mass spectrum clearly indicate that the compound is the one postulated.

The amounts of *N*-carbethoxyproline and *N*-carbethoxyglycine formed in the model wine solutions were determined by extracting 100-ml portions of 3.5 buffer containing the carbethoxy derivative at 0, 100, 500, and 1000 μg/l. The peak–area responses for the methylated extracts as detected by the GLC–Coulson system were linear. Multiple GLC–Coulson injections gave as much as ±3.5% difference in area. Area measurements by the planimeter varied as much as 1–3%. For the purpose of this study, no internal standards were considered necessary. Any suitable amino acid not present in abundance in wine could be derivatized and added as an internal standard. An accuracy of at least ±10% is estimated for *N*-carbethoxyproline and -glycine in the range of 100 to 1000 μg/l.

Model Solutions. Because of the reactivity of DEPC it was necessary to determine the parameters of the reactions under controlled conditions. Model wine solutions were prepared at several pH values and treated with controlled amounts of DEPC and amino acids. The samples were analyzed. Figure 5 plots the data on a weight basis. Table I gives the production of *N*-carbethoxy derivatives as a function of DEPC concentration.

Table II. Diethyl Pyrocarbonate Treatment of Carbernet and Chardonnay Wines

Sample	*N*-Carbeth-oxyproline, mg/l.
Cabernet (untreated)	0
Cabernet + 0.25 mg of *N*-carbethoxyproline	1.0
Cabernet + 100 mg/l. of DEPC (extraction 1)	1.65
Cabernet + 100 mg/l. of DEPC (extraction 2)	1.7
Chardonnay (untreated)	0
Chardonnay + 0.25 mg of *N*-carbethoxyproline	1.0
Chardonnay + 100 mg/l. of DEPC (extraction 1)	2.3
Chardonnay + 100 mg/l. of DEPC (extraction 2)	2.5

Table III. Effect of pH and Proline Concentration on the *N*-Carbethoxyproline Production in Wines

Wine	pH	Proline, mg/l.	*N*-Car-bethoxy-proline, mg/l.
Grenache	3.2	630	1.5[a]
Petite Sirah	3.5	360	1.05[a]
Grey Riesling	3.76	1140	5.2[a]
Carignane	3.0	160	0.3
French Colombard	3.3	1740	4.5
Chardonnay	3.1	1240	2.4[a]
Cabernet Sauvignon	3.3	660	1.7[a]

[a] Average of duplicate extractions.

The nonlinearity of reaction to variations in pH (Figure 5) can be explained (Mahler and Cordes, 1971). The model they propose is in good agreement with these experimental data. Table I shows that the amount of DEPC added is directly proportional to the concentration of the product formed. Similarly, the amount of amino acid present is proportional to the amount of product formed.

Wines. The analytical results of one red and one white wine are given in Table II. The efficiency of extraction of *N*-carbethoxyproline added to wine is 90% for the Cabernet Sauvignon and 96% for the Chardonnay as compared with extraction from model wine solution. Duplicate extractions differed by no more than 5%, rivaling the replicability of injections of the same samples. In the area of the *N*-carbethoxyglycine or -proline methylated products no other peak was found. Natural glycine is at such a low level it is not seen as a derivative when DEPC is added to the wine. Table III shows the pH and proline concentrations of the treated wines as well as the results of the tests. Figure 5 plots these final data for comparison with model–wine reaction products.

Assuming an error range of 10–15% for single replications of the method, the extractions can be considered essentially complete. Subsequent single extractions of the products from wine compared with single methylations of pure standards gave efficiencies varying from 85 to 110%. The failure to find a peak for the *N*-carbethoxyproline (or -glycine) without DEPC treatment of the wine does not rule out the possibility that one was present. The sensitivity of the method was in the range of 25–50 μg/l. The treatment of wine produced only two-thirds as much as is produced in the model wine. Other reactions going on probably account for this loss.

Some further work discussing the enzymatic fate of these two compounds is discussed in another article (Baker and

Ough, 1976).

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Trace Element Analysis of Wine by Proton-Induced X-Ray Fluorescence Spectrometry

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Using proton-induced x-ray fluorescence spectrometry (PIX), nine trace elements have been simultaneously determined in representative wines from the San Joaquin Valley of California. With the exception of high copper content in two muscatels, all wines had low concentrations of the elements examined, and were within the ranges previously reported in wines. On the basis of these nine wines, no conclusions can be made as to patterns or trends of elemental composition between location sites or wine types, but further investigation by PIX, which is very well suited for wine analysis, seems warranted.

Trace element analysis of wine has long been of interest and concern to enologists. The deleterious effects on color, aroma, and taste of several metals, including copper, iron, zinc, nickel, tin, and aluminum, have been reviewed by Mrak et al. (1937), Mrak and Fessler (1938), Amerine (1958), Amerine et al. (1972), and Eschnauer (1974). Copper and iron, which contribute to haze formation and color defects in wine (Berg and Akiyoshi, 1956; Amerine et al., 1972), are monitored routinely as part of standard wine quality control tests. Nickel has also been shown to cause clouding in wines (Eschnauer, 1965b).

The trace element composition of grapes is influenced by the soil, by equipment used during vinification, and by wine processing treatments such as fining or filtration. It is further affected by the trace metals in insecticides, fungicides, or nutrients used in the vineyard and by environmental pollution. Because of concern over potentially toxic elements such as Pb, As, or Cd, these elements have also been surveyed in wine (Rankine, 1955; Eschnauer, 1965a; Edwards, 1973; Martina et al., 1973; Basile and Tarallo, 1974; Castelli et al., 1974; Garrido et al., 1974). Typical concentration ranges for selected elements in wines are shown in Table I. Maximum acceptable levels in wine for several metals as proposed by the Office International de la Vigne et du Vin (O.I.V.) for its member countries and by Canada and Germany are shown in Table II. More extensive reviews of the inorganic constituents in wine have been published by Amerine (1958) and Eschnauer (1974).

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Determination of the content of any metal has typically been done by time consuming element-specific procedures. Amerine (1958) and Schneyder (1974) have surveyed the chemical determination procedures and atomic absorption techniques used in wine trace metal analyses. Because of the interest in the quantitative determination of several elements, such as copper and iron for wine stabilization control and lead or arsenic to monitor pollutants, the use of a multielement technique for the simultaneous analysis of many elements is desirable in wine analysis. Energy-dispersive x-ray fluorescence analysis has the advantage over its principal competitor, neutron activation analysis, that the elemental sensitivity is a smoothly varying function of atomic number Z. The proton-induced variant of x-ray fluorescence analysis (PIX) has its maximum sensitivity in the regions $25 < Z < 35$, which includes Cu, Fe, Zn, Ni, As, and $Z \sim 80$, including Pb. In this method accelerated charged particles serve to induce fluorescent x rays, the energy spectrum of which is then analyzed by a Si(Li) detector to identify and quantify the elements present. The choice of physical parameters for optimizing PIX analyses has been discussed by Herman et al. (1973) and application to analysis of biological materials is dealt with in detail by Campbell et al. (1975).

Wine is ideally suited for trace metal analysis by this technique. The elements are present in high enough concentrations to permit direct sampling of the wine without preliminary concentration steps. Furthermore, as a homogeneous and aqueous system, neither acid digestion nor ashing, which are generally required by atomic absorption procedures, is required for sample preparation. However, even in spotting a homogeneous solution such as wine, different thicknesses in the target may be produced and quantification assuming uniform cross-sectional