Engels, C.; Hendrick, M.; DeSamblaux, S.; DeGryze, I.; Tobback, P. Modelling Water Diffusion Long-grains Rice Soaking. *J. Food Eng.* **1986**, *5*, 55–73.
Fan, L.; Chung, D. S.; Shellenberger, A. J. Diffusion Coefficients of Water in Wheat Kernels. *Cereal Chem.* **1961**, *38*, 540–548.
Haishi, T. Basic Modes of Water Absorption by Woods. *Mokuzai Gakkaishi* **1980**, *26*, 510–518.
Hsu, K. H. Effect of Temperature on Water Diffusion in Soybean. *J. Food Sci.* **1983**, *48*, 1364–1365.
Nikolov, S.; Abrashev, G.; Katsarova, V. Osobenosti pri Nabbaneto na Drvesinata v Tekhnosti. *Ser. Mekh. Tekhnol. Durv.* **1979**, *24*, 47–51.
Schmidt, K. Untersuchungen uber die Raumklimapufferung durch Holzoberflächen. *Holzforsch. Holzverwert.* **1986**, *38*, 1–3.
Steffe, J. F.; Singh, R. P. Diffusivity of Starchy Endosperm and Bran of Fresh and Rewetted Rice. *J. Food Sci.* **1980a**, *45*, 356–361.
Steffe, J. R.; Singh, R. P. Note on Volumetric Reduction of Short Grain Rice During Drying. *Cereal Chem.* **1980b**, *57*, 148–150.
Suarez, C.; Viollaz, P.; Chirife, J. Diffusional Analysis of Air Drying of Grain Sorghum. *J. Food Technol.* **1980**, *15*, 523–531.
Sumi, H. High Temperature Drying of Wood. *Mokuzai Gakkaishi* **1978**, *24*, 385–390.
Zhang, T. Y.; Bakshi, A. S.; Gustavson, R. J.; Lund, D. B. Finite Element Analysis of Nonlinear Water Diffusion During Rice Soaking. *J. Food Sci.* **1984**, *49*, 246–250.

Received for review September 30, 1988. Accepted February 15, 1989.

Urea Analysis for Wines

John Almy[1] and Cornelius S. Ough*

An analysis for urea in wine was developed in which urea is isolated by ion exchange and then derivatized with 1-phenyl-1,2-propanedione 2-oxime. Measurements of the derivative are made by colorimetry. The analysis gave good linear response to additions of 0–50 mg/L of urea to either red or white wine. Blank experiments in which either urea or 1-phenyl-1,2-propanedione 2-oxime was withheld with either red or white wine samples or model solutions gave less than 1.0 mg/L of urea in all cases. Good agreement was obtained for wine samples that were submitted to the present method and one to HPLC.

Wines and other fermented beverages can occasionally have relatively high levels of ethyl carbamate (a carcinogen). Studies by Ough et al. (1988a) have determined that urea reacting with ethanol is the major cause of formation of ethyl carbamate in wines. This has been verified by tracer work (Bisson, personal communication). Citrulline present in wines can also contribute to ethyl carbamate formation by reacting with ethanol. Ough et al. (1988b) reported the Arrhenius data for both urea and citrulline for their reaction with ethanol. Both urea and citrulline are found in wines at about the same amount, but urea reacts to a much greater extent than does citrulline. Ough et al. (1989) and Caputi (personal communication) found that heavily fertilized vineyards were probably the major cause of high urea remaining in the wine because of high nitrogen nutrients in the juice. The yeast strain and fermentation temperature can also cause increased urea accumulation, resulting in high-potential ethyl carbamate formation (Ough, unpublished work). The need for a quick and reasonably accurate urea method to screen wines for high urea content was enhanced by the report of Yoshizawa and Takahashi (1988), which indicated that an acid urease, active in sake and in wine, was being developed commercially. In addition, the Food and Drug Administration has indicated a need to maintain low levels of ethyl carbamate in wines.

Wine contains ammonia in concentrations high enough to interfere with the enzymatic method of urea determination. Wine also contains sugars that would interfere with colormetric methods unless they were first removed. For these reasons, we sought to develop a simple method for wine that would measure urea at the 1–50 mg/L range.

Department of Viticulture and Enology, University of California, Davis, California 95616.
[1] Permanent address: Department of Chemistry, California State University, Stanislaus, Turlock, CA 95380.

EXPERIMENTAL SECTION

Reagents. Urea (Sigma, St. Louis, MO), 1-phenyl-1,2-propanedione 2-oxime (Aldrich, Milwaukee, WI—also known as α-isonitrosopropiophenone), and other reagents were purchased and used without further purification. Deionized water was used as solvent unless otherwise specified.

Ion-exchange columns were prepared by loading 0.90 g of Dowex 50W-X8, 50–100 mesh, onto 5 mm (i.d.) × 90 mm glass columns with frits (Bio-Rad, Richmond, CA, Catalog No. 737-0510); these columns were prewashed and regenerated after use with 7 mL each of water, ethanol, water, 2 M HCl (14 mL), water, 1.5 M NaOH (14 mL), water, and 0.1 M HCl (21 mL).

Chromatography, Derivatization, and Measurement. A mixture of 2.0 mL of the wine or calibration sample and 0.32 mL of 1.0 M aqueous HCl was placed onto the column. Two 1.5-mL portions of 0.1 M HCl and then 1.5 mL of 4 M NaCl in 0.1 M HCl were added. The receiver was then changed, and the urea-containing fraction was eluted with 3.7 mL of 4 M NaCl in 0.1 M HCl. Two 1.75-mL portions of this fraction were placed in separate 16 mm (o.d.) × 150 mm culture tubes. To one tube was added 0.100 mL of a solution of 0.200 g of 1-phenyl-1,2-propanedione 2-oxime in 5.0 mL of ethanol, and to the other (the blank) was added 0.100 mL of ethanol. Each tube was vortexed, and 1.25 mL of a 1:3:1.25 (by volume) mixture of 18 M H_2SO_4–14 M H_3PO_4–H_2O was added. The tubes were vortexed again, sealed with PTFE-lined screw caps, placed in boiling water for 2 h without light, and then brought to room temperature over 30 min in the dark.

Colorimetric measurements of optical density to the nearest thousandth of an absorbance unit at 540 nm were made on a Bausch and Lomb Spectronic 710 instrument equipped with a micro flow-through system, a microcell, and a digital readout. In

0021-8561/89/1437-0968$01.50/0 © 1989 American Chemical Society

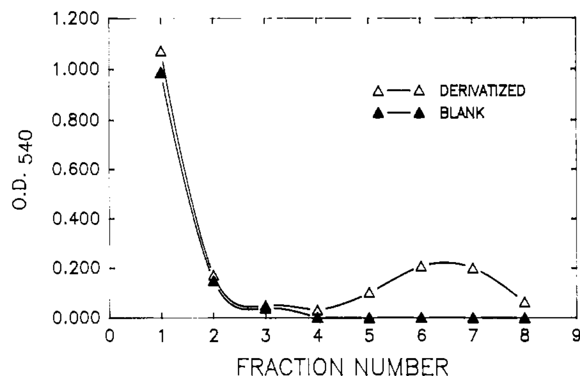

Figure 1. Fractionation of Muscat wine by cation-exchange chromatography. Each fraction was divided into derivatized (Δ) and blank (▲) samples. Optical density measurements are plotted against the fraction number.

all cases, the blank readings were subtracted from the readings from samples treated with 1-phenyl-1,2-propanedione 2-oxime. Except for red wines less than 2 years old, blank readings for wine samples were essentially the same as those from standard samples. Samples giving optical density readings over 0.7 were rerun after dilution with water. Dilutions of calibration standards containing over 35 mg/L were similarly made. Replicate data are reported with ±1 standard deviation.

RESULTS AND DISCUSSION

Chromatography and Derivatization Procedure. After some trials, a modified method of Shelp et al. (1985) was found to remove substances in wine that interfered with the colorimetric measurements. In order to establish the ideal cleanup procedure, fractionation of a wine sample on a prepared column was run using only 0.1 M HCl eluant. Following the introduction of the wine, 1-mL fractions were taken, diluted to 3.7 mL, divided evenly into reaction tubes, and analyzed (see the Experimental Section). Figure 1 shows the data for a Muscat wine sample (ca. 8% sugar and 14% alcohol): The presence of an interfering substance, probably sugar, is indicated in the first three fractions. After the fourth fraction, the derivatized series indicates the presence of urea free of interferences. The amounts of dilute HCl and NaCl–HCl solutions used for removal of interfering substances were optimized by trials on red, sweetened wine; in the present case, 3.0 mL of dilute HCl and 1.5 mL of NaCl–HCl solutions led to the highest amounts of urea in the measured fraction.

With use of the above method, two series of standard data were obtained for additions of 0, 5, 10, 20, and 50 mg/L of urea. In one series, these amounts were added to pH 3.2 (0.2 M phosphate) buffer containing 12% (v/v) ethanol; the resulting optical density versus milligrams per liter plot yielded a linear correlation by least-squares best fit whose slope, intercept, and correlation coefficient (R) values were 0.030 AU·L·mg^{-1}, 0.003 AU, and 0.998, respectively. For the other series, these measured amounts were added to red wine; the same parameters for the resulting line were 0.032 AU·L·mg^{-1}, 0.024 AU, and 0.999, respectively.

For red wines less than 2 years old, pigments were both eluted before the urea and retained while urea was collected. A small amount of pigment coeluted with urea, but the color was almost completely extinguished by the hot strong-acid medium of the derivatization step. Typical blank readings for these samples would have made the urea values higher by 0.5–0.9 mg/L had they not been subtracted. Older red and all white wines seldom gave blank values higher than 0.002.

Wine that had been adjusted to pH 4 (NaOH) and exposed to urease for 1 week at room temperature gave an

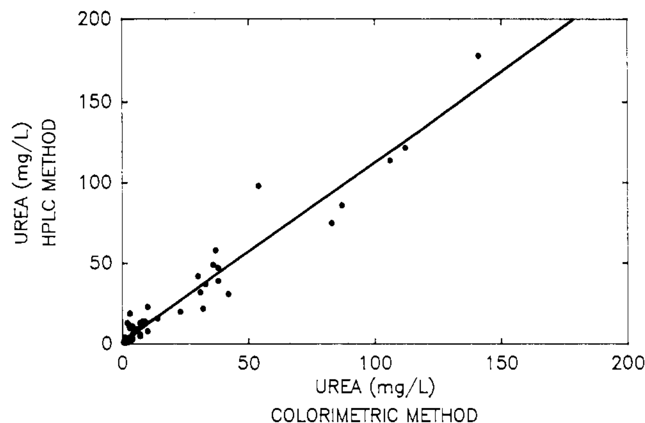

Figure 2. Comparison of measurements on 39 wine samples using the HPLC–ninhydrin method (vertical axis) against this method.

analysis of 0.1 mg/L of urea.

Citrulline (present in wine at ca. 25 mg/L) participates in the urea cycle and could produce urea under the conditions of the analysis. Therefore, interference by this compound was tested: To a wine that had previously measured 9.0 ± 1.0 mg/L of urea was added 100 mg/L of citrulline; the analysis then gave 9.6 ± 0.2 mg/L of urea.

Recovery, based on a comparison of colorimetric readings from standard solutions that were submitted to the chromatography procedure to those for the same solutions placed directly into the reaction tubes, averaged 80%. An experiment in which a 10 mg/L of urea sample was heated as a blank for 2 h and then heated for an additional 2 h with 1-phenyl-1,2-propanedione 2-oxime gave an absorbance essentially equal to that for the same sample derivatized and heated for only 2 h. This indicates that little or no loss of urea occurred during the derivatizing step.

Comparison of the Present Method with Measurement by the HPLC–Ninhydrin Method. Figure 2 compares the data obtained by the method described above and a HPLC method of Burns (personal communication), which is accurate to about 5 mg/L. A least-squares line for this plot had a slope of 1.1, an intercept of 2.5, and a R value of 0.96. For samples above 10 mg/L, this method and the HPLC method gave relative standard deviations of 6.2% and 7.2% respectively, while for samples below 10 mg/L these relative standard deviations were 14.9% and 53.8%, respectively.

Since the development of the method, well over 100 new wines have been analyzed for urea and then heated to determine the potential ethyl carbamate. The preliminary results indicate that the method of screening new wines (with no detectable ethyl carbamate prior to heating) works satisfactorily.

CONCLUSION

The procedure described in this study is rapid, inexpensive, and accurate enough to identify wine samples that are likely to give high levels of ethyl carbamate after aging or by rapid aging heat treatments.

ACKNOWLEDGMENT

We thank Tali Sendovski for her assistance in analyzing many of the samples.

Registry No. Urea, 57-13-6; 1-phenyl-1,2-propanedione 2-oxime, 119-51-7.

LITERATURE CITED

Ough, C. S.; Crowell, E. A.; Mooney, L. A. Formation of ethyl carbamate precursors during grape juice (Chardonnay) fermentation. I. Addition of amino acids, urea and ammonia: Effects of fortification on intracellular and extracellular pre-

cursors. *Am. J. Enol. Vitic.* **1988a**, *39*, 243–249.
Ough, C. S.; Crowell, E. A.; Gutlove, B. R. Carbamyl compound reactions with ethanol. *Am. J. Enol. Vitic.* **1988b**, *39*, 239–242.
Ough, C. S.; Stevens, D.; Almy, J. Research note: Preliminary comments on effects of grape vineyard nitrogen fertilization on the future ethyl carbamate formation in wines. *Am. J. Enol. Vitic.* **1989**, in press.
Shelp, B. J.; Sieciechowicz, K.; Ireland, R. J.; Joy, K. W. Determination of urea and ammonia in leaf extracts: application to ureide metabolism. *Can. J. Bot.* **1985**, *63*, 1135–1140.
Yoshizawa, K.; Takahashi, K. Utilization of urease for urea decomposition in sake. *J. Brew. Soc. Jpn.* **1988**, *83*, 142–144.

Received for review October 14, 1988. Accepted March 9, 1989.

Ruminal Organic Acid Analysis by Gas Chromatography/Mass Spectrometry[1]

Sergio Daolio,* Mario Bonsembiante, Giovanni Bittante, Maurizio Ramanzin, and Piero Rinaldo

Rumen fluid was analyzed by the gas chromatography/mass spectrometry (GC/MS) technique in order to identify the organic acids contained. The gas chromatogram obtained showed more than 200 peaks, and 60 organic acids were identified from their mass spectra obtained under electron impact conditions from the relative chromatographic peaks. Keto acids, polycarboxylic acids, hydroxy acids, aromatic acids, and saturated and unsaturated fatty acids were present, which were subdivided into three main groups: (i) short- and long-chain fatty acids; (ii) polyfunctional organic acids such as intermediate metabolic products; (iii) phenolic acids mainly from lignin and tannin degradation. It was concluded that GC/MS is a very specific and sensitive technique to detect the presence of fermentation products in biological fluids and that it could allow for the simpler and cheaper GC technique to be used for routine quantitative analyses of the identified compounds.

Research on ruminal fermentation of feedstuffs is mainly based on measurements of pH, volatile fatty acids concentration, and turnover of markers. A more analytical approach is limited by difficult laboratory procedures regarding the identification and quantitation of the different biochemical pathways.

We have recently used mass spectrometry/mass spectrometry (MS/MS) techniques to analyze the gas produced during the fermentation in the rumen (Bonsembiante et al., 1987a,b), and we have also adapted the MS/MS (McLafferty, 1983) and the gas chromatography/mass spectrometry (GC/MS) (McFadden, 1973) techniques to cope with the mixture analysis in order to study the chemical changes of ensiled grass (Bonsembiante et al., 1985; Daolio et al., 1986).

The present work extends the method to analyze the nonvolatile organic acid profile of rumen fluid and to obtain the mass spectra of each component of the mixture.

This basic research is a preliminary but essential step in order to carry out a complete compound screening and to perform routine quantitative analyses with a less sophisticated technique such as gas chromatography.

MATERIALS AND METHODS

Sample Preparation. The rumen fluid (3 mL) was drawn from a wether sheep fitted with a rumen cannula and fed hay and limited amounts of concentrate, deproteinized with ethanol (18 mL) and then alkalinized to pH 14 with NaOH 30% and extracted twice with an equal volume of ethyl acetate and once with diethyl ether. The aqueous phase was submitted to the following preparative steps: (i) oximation of α-keto acids with hydroxylamine hydrochloride (Adibi, 1976); (ii) silylation of carboxylic and hydroxylic functional groups with *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) and trimethylchlorosilane (TMCS) as a catalyst (Pierce, 1968).

Table I. Ionic Fragments and Their Relative Abundances Obtained from Peak 79 of the Chromatogram (Identified as 2-Hydroxyglutaric Acid)

ionic fragment, m/z	rel abund, %	ionic fragment, m/z	rel abund, %	ionic fragment, m/z	rel abund, %
32	7	75	23	157	23
43	7	85	12	203	37
44	7	129	100	294	7
45	21	130	13	231	10
55	6	131	7	247	47
69	6	133	7	248	10
73	78	147	75	249	5
74	7	148	7	349	7
				350	2

The derivatized organic acids are very stable and can be run under the chromatographic conditions.

However, short-chain aliphatic acids that do not contain additional functional groups can be lost during the preparative steps or coelute with the solvent and reagents used. These low molecular weight acids are then extracted by steam distillation and analyzed by the GC technique.

Analysis. *Apparatus.* The qualitative analysis was performed by means of a HP 5792A gas chromatograph coupled with a HP 5970A mass spectrometer and a HP 9825B data acquisition system. The operative instrumental conditions were selected as follows: (i) SE 52 fused silica capillary column, 25-m length; (ii) sample volume introduced for every analysis, 1 μL (split mode 1:30); (iii) helium flow, 1.5 mL/min; (iv) injector temperature, 250 °C; (v) interface and FID detector temperatures, 275 and 300 °C, respectively (instrument is equipped with conventional FID

Istituto di Polarografia ed Elettrochimica Preparativa del CNR, Corso Stati Uniti 4, 35100, Padova, Italy (S.D.), Istituto di Zootecnica, Facoltà di Agraria, Via Gradenigo 6, 35131, Padova, Italy (M.B., G.B., M.R.), and Dipartimento di Pediatria, Università di Padova, Via Giustiniani 3, 35100, Padova, Italy (P.R.).

[1] Research supported by the CNR, Italy. Special Grant IPRA. Paper No. 1514.

0021-8561/89/1437-0970$01.50/0 © 1989 American Chemical Society