for one analysis is comparable with that of HPLC–UV methods. However, it takes about 3 days to produce the results, because of the relatively long incubation time of the CPB assay.

Registry No. Vitamin D, 1406-16-2.

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Received for review May 7, 1985. Revised manuscript received September 16, 1985. Accepted October 9, 1985.

Analysis of Methoxypyrazines in Wines. 1. Development of a Quantitative Procedure

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A new method for the quantification of 2-methoxy-3-isobutylpyrazine (3IBP) from wine was developed. Wine was spiked with both 3IBP and 2-methoxy-3-isopropylpyrazine (IPP) and extracted by steam distillation. The distillate was adsorbed onto a C-18 SEP-PAK cartridge with subsequent methanol elution. This concentration step was followed by analysis of the eluant by reversed-phase high-pressure liquid chromatography (HPLC). The recovery for 3IBP was 52.9 ± 7% with a minimum detection level of 1.2 μg/L (assuming 50% recovery). For IPP the recovery was extremely inefficient with only 14 ± 4.7% recovered. As demonstrated herein, light must be excluded from the analysis. After exposure to light for 120 h, aqueous solutions of either methoxypyrazine showed a loss of approximately 28% by photodegradation, whereas no change occurred in samples from which light was excluded.

Pyrazines are found in a large number of foods and other natural products. Most alkylpyrazines are formed by Maillard reactions via Strecker degradation when foods are heated, although some are produced by microorganisms such as *Saccharomyces cerevisiae* in beer and wine (Maga, 1982; Kosuge et al., 1971). These compounds are partly responsible for the pleasant aromas of roasted meats, coffee, cocoa, and cereals (Maga, 1982). Alkoxypyrazines are found in raw vegetables such as peas, bell peppers, potatoes, beets (Murray and Whitfield, 1975), and grapes (Bayonove et al., 1975; Augustyn et al., 1982). These generally "vegetative" or musty, earthy compounds are produced as contaminants in water, milk, and other fluids mainly by *Actinomyces spp.* (Mottram et al., 1984; Gerber, 1979; Morgan, 1976; Miller et al., 1973).

The alkoxypyrazine 2-methoxy-3-isobutylpyrazine (3IBP) was first isolated from bell peppers by using a simultaneous distillation–extraction technique (SDE). This compound, which has the characteristic odor of bell peppers, is a potent odorant; in water it has an odor detection threshold of 2 parts in 10^{12} (Buttery et al., 1969). Bayonove et al. (1975) were the first to tentatively identify 3IBP in Cabernet Sauvignon grapes using a pentane liquid–liquid extraction. However, using a methylene chloride extraction, Slingsby et al. (1980) were unable to detect any 3IBP in a Cabernet Sauvignon wine that had a distinctly vegetative aroma. In addition to 3IBP, 2-methoxy-3-isopropyl- and 2-methoxy-3-*sec*-butylpyrazine were tentatively isolated from Sauvignon blanc grapes by using Freon extraction and headspace techniques (Augustyn et al., 1982). All of these workers only qualitatively analyzed their systems, reporting only whether the compound was detected or not.

Using a headspace technique to determine the approximate levels of the methoxypyrazines, the first quantitative data were collected by Murray and Whitfield (1975) for a variety of vegetables. For this analysis, they claimed "no great accuracy". The only reproducibility or recovery data for analysis of the alkoxypyrazines are those reported by Krasner et al. (1983). Using a closed-loop stripping method followed by gas chromatography, 84 ± 18% IPP and 102 ± 31% 3IBP were recovered from spiked water samples. However, this method is not appropriate for analysis of methoxypyrazines in complex systems such as wine. In

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0021-8561/86/1434-0268$01.50/0

the present study, methoxypyrazines were isolated by steam distillation, concentrated by adsorption onto a C-18 SEP-PAK, and analyzed by high-pressure liquid chromatography (HPLC). The recovery and reproducibility of the technique were demonstrated by analysis of two alkoxypyrazines added to white wine.

MATERIALS AND METHODS

Apparatus. HPLC analyses were performed on a Waters system consisting of a system controller, a data module, and a pump (Waters Associates, Milford, MA 01757) using a UV spectrophotometer (Perkin-Elmer LC-55-B, Norwalk, CN 06856) at 280 nm (maximum sensitivity) as the detector. A Brownlee RP-18 Spheri-5 column (4.6 mm i.d. × 22 cm) (Brownlee Laboratories, Santa Clara, CA 95050) connected to a Brownlee RP-8 Spheri-5 guard column (4.6 mm × 3 cm) was used. The mobile phase was 72% aqueous methanol buffered with 0.01 M $(NH_4)H_2PO_4$ and adjusted to pH 2.6 with concentrated phosphoric acid. A flow rate of 0.8 mL/min was used to keep the pressure below 3000 psi. The 100- or 200-μL injections were made on an autosampler (Waters Associates).

Reagents. All reagents were laboratory grade. The methanol used for the HPLC liquid phase was redistilled in glass and degassed daily. The pyrazines evaluated were tetramethylpyrazine (TMP), 2-methoxy-3-isopropylpyrazine (IPP), and 2-methoxy-3-isobutylpyrazine (Aldrich Chemical Co, Milwaukee, WI 53201). As supplied, the 2-methoxy-3-isobutylpyrazine contained 28.3% 2-methoxy-6-isobutylpyrazine (6IBP) and 71.7% 3IBP (as specified by Aldrich). A chenin blanc wine (12.4% v/v ethanol) made in the UCD experimental winery was used for the reproducibility and recovery determinations.

Analysis of Standards. Preliminary studies indicated that the aqueous TMP standard solutions were stable over time but that the methoxypyrazine solutions were not. Loss by volatilization seemed improbable, since vials were sealed with Teflon septa. These studies also showed that the loss was not due to adsorption on glass. Hence, suggestions that pyrazines might be light sensitive prompted an evaluation of the effect of light on methoxypyrazines. Standard solutions were made in double-distilled deionized water (DDW) adjusted to pH 2.2 with concentrated sulfuric acid and analyzed over a period of 120 h. Samples were held in 4-mL glass WISP vials covered with aluminum foil to exclude light or uncovered under fluorescent light (44 μEinstein m^{-2} s^{-1}). At selected intervals, two vials from each light condition were analyzed in triplicate by HPLC.

Wine Analysis. Since grape juice and wines contain nonvolatile phenolic material that coelute with the pyrazines under the the chromatographic conditions employed, the pyrazines were recovered from wine by steam distillation. For steam distillation a 3-L steam generator and a 5-L sample flask were utilized. The 2-L receiving flask, immersed in an ice bath (0 °C) was connected to the sample flask by two condensers in series. A water-cooled condenser (19 °C) was connected to the sample flask, and an ethylene glycol cooled condenser (1.6 °C) was connected to the receiving flask inlet. Another glycol-cooled condenser (1.6 °C) was attached to the outlet of the receiving flask. The inlet frit to the receiving flask was immersed in 100 mL of 0.1 N HCl solution. The 500-mL sample (model solution or wine) was adjusted to pH 5 with 4 N NaOH immediately prior to distillation.

The sample was steam distilled for 40 min, which had been shown in previous trials to provide optimum pyrazine recovery. At the end of the 40-min distillation, pyrazines in the 840 ± 35 mL distillate were concentrated on a preactivated SEP-PAK C-18 cartridge (Waters Associates). The distillate was pushed through the SEP-PAK in 50-mL aliquots with a variable-speed syringe pump (Sage Instruments Model 355) at a rate of aproximately 30 mL/min. Pyrazines were eluted from the Sep-PAK with 2 × 1 mL methanol aliquots and brought to 5 mL with DDW. Samples were placed in covered vials to exclude light. Each sample was analyzed by HPLC in triplicate.

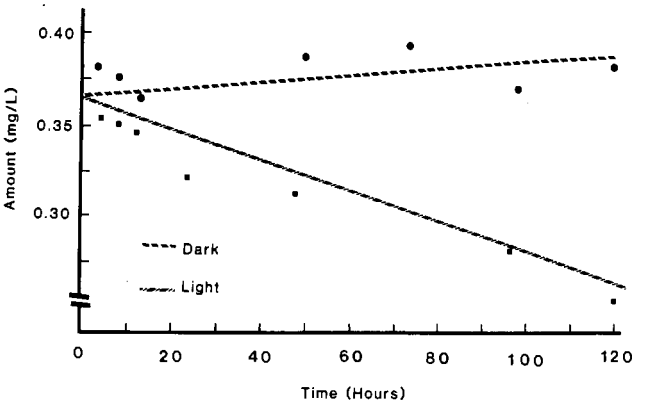

Figure 1. Change in the mean concentration of aqueous 2-methoxy-3-isopropylpyrazine (IPP) standards held in the dark (●) and the light (■) over time. Initial concentration 0.522 mg/L (n = 6).

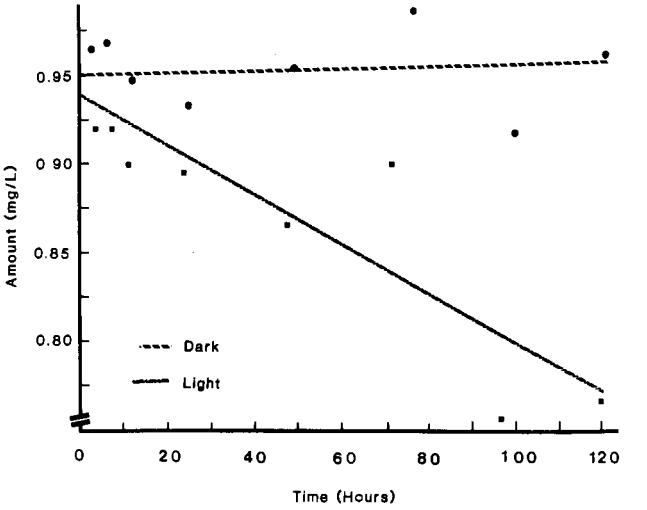

Figure 2. Change in the mean concentration of aqueous 2-methoxy-3-isobutylpyrazine (3IBP) standards held in the dark (●) and the light (■) over time. Initital concentration 0.954 mg/L (n = 6).

Data Analysis. The time study results were analyzed by simple regression. Average response factors for the standards were calculated as were standard deviations. Response factors are defined as the amount of compound (μg) divided by the peak area. The mean percentage recovery of the methoxypyrazine standards from spiked wines was also determined by using the average response factors of the methoxypyrazines as determined by HPLC.

RESULTS AND DISCUSSION

Aqueous solutions of TMP were stable over time as shown by the constant response factor for TMP over 120 h: 0.0027 ± 0.000 17 (n = 68), with a coefficient of variation of 6.2%. Similarly, 3IBP and IPP held in the dark were stable over time. However, the concentration of IPP and 3IP exposed to light decreased over time, as indicated by an increase in response factor. Figures 1 and 2 show the decrease in methoxypyrazine concentrations in light-exposed solutions for IPP and 3IBP, respectively, in contrast

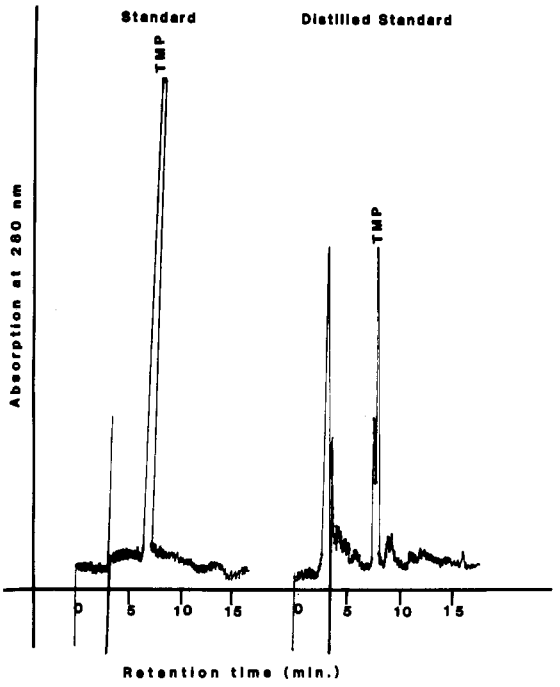

Figure 3. Typical high-pressure liquid chromatograms: (a) an aqueous tetramethylpyrazine (TMP) standard; (b) steam distillate of TMP and degradation products.

to the stable samples held in the dark. As shown in Table I, the slopes of the concentration–time functions for standard solutions in the dark and in the light differed significantly for both compounds. Over the 120 h of light exposure, approximately 28% loss of either compound resulted from the photodegradation.

Use of the light-stable TMP as an internal standard in the distillations to calibrate the recoveries of the methoxypyrazines was attempted. However, the TMP was unstable under the steam distillation conditions. In all steam distillations of TMP, in addition to TMP, unknown products that were also UV absorbent were recovered and separated as shown in Figure 3.

Table I. Regression Equations for the Methoxypyrazines Held in the Dark and in the Light (n = 9)

equation	correl (r)	signif[a]
2-Methoxy-3-isopropylpyrazine[b]		
dark $Y = 3.58 \times 10^{-5}$(time) + 0.515	0.665	ns
light[c] $Y = -7.05 \times 10^{-4}$(time) + 0.510	0.934	***
2-Methoxy-3-isobutylpyrazine[b]		
dark $Y = 3.85 \times 10^{-5}$(time) + 0.950	0.077	ns
light $Y = -1.40 \times 10^{-3}$(time) + 0.915	0.822	**

[a] ns, **, ***: not significant, $p < 0.01$, $p < 0.001$, respectively. [b] Slopes between light and dark functions differed significantly ($p < 0.001$). [c] n = 8.

Table II. Recovery of 2-Methoxy-3-isobutylpyrazine (3IBP) and 2-Methoxy-3-isopropylpyrazine (IPP) from a Spiked White Wine

amt added to wine prior to distilln, μg	amt[a] recd from distilln, μg	rec eff, %
	3IBP	
1.88	0.98	52
1.83	1.09	59
1.71	1.06	62
1.35	0.79	56
1.35	0.60	45
3.11	1.34	43
3.02	1.61	53
		52.9 ± 7[b]
	IPP	
2.25	0.42	19
2.25	0.37	17
3.42	0.35	10
3.30	0.32	10
		14 ± 4.7[c]

[a] Average of three HPLC analyses. [b] Overall mean, n = 6. [c] Overall mean, n = 4.

In Table II the recoveries of IPP and 3IBP from a spiked white wine are shown. The unspiked wine (Figure 4a) had no UV absorbance at the retention times of the meth-

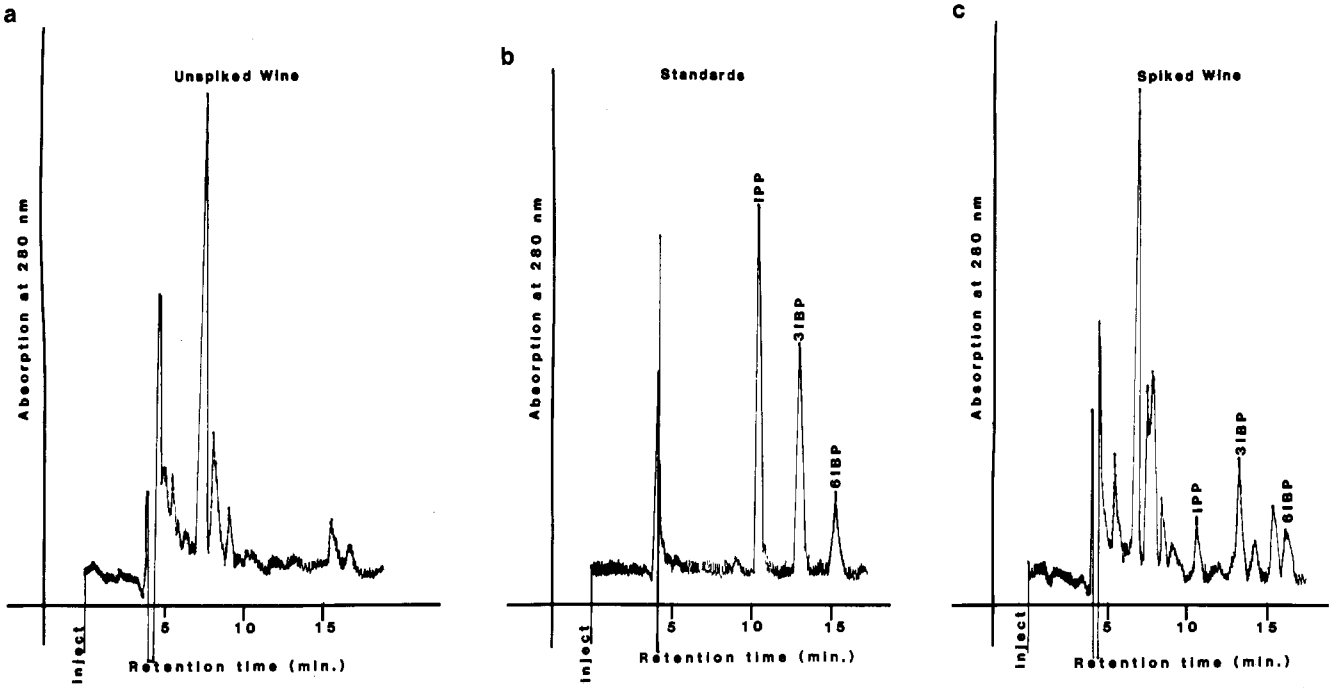

Figure 4. High-pressure liquid chromatograms: (a) steam distillate of base white wine; (b) aqueous standards of 3-methoxy-2-isopropyl- (IPP), 3-methoxy-2-isobutyl- (3IBP), and 3-methoxy-6-isobutylpyrazine (6IBP); (c) steam distillate of base white wine with added methoxypyrazines.

Table III. Retention Times of Pyrazines on a C-18 Reversed-Phase Column[a]

pyrazine	ret time,[b] min	pyrazine	ret time,[b] min
tetramethyl	7.3	2-methoxy-3-isobutyl	13.3
2-methoxy-3-isopropyl	10.7	2-methoxy-6-isobutyl	15.5

[a] Mobile phase: 72% aqueous methanol buffered with 0.01 M $(NH_4)H_2PO_4$ and adjusted to pH 2.6 with concentrated phosphoric acid. [b] Flow rate 0.8 mL/min.

oxypyrazines standards (Figure 4b; Table III). In the wine spiked with methoxypyrazines, several peaks were observed near the retention time of TMP; some of them may have been TMP and the TMP breakdown unknowns (Figure 4c), as TMP has been previously reported in wines by Kosuge et al. (1971). Recovery of the 3IBP ranged between 43 and 62%. Assuming 50% recovery, the minimum detection level by this technique is 1.2 μg/L. In contrast, the recovery of IPP was extremely inefficient, in all cases being less than 19%.

ABBREVIATIONS USED

SDE	simultaneous distillation–extraction
3IBP	2-methoxy-3-isobutylpyrazine
TMP	tetramethylpyrazine
IPP	2-methoxy-3-isopropylpyrazine
6IBP	2-methoxy-6-isobutylpyrazine
DDW	double-distilled water

ACKNOWLEDGMENT

We thank T. C. Somers for helpful information.

Registry No. IPP, 25773-40-4; 3IBP, 24683-00-9.

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Received for review February 8, 1985. Revised manuscript received October 7, 1985. Accepted December 6, 1985.

Simultaneous Analysis of Ascorbic and Dehydroascorbic Acid by High-Performance Liquid Chromatography with Postcolumn Derivatization and UV Absorbance

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A high-performance liquid chromatography (HPLC) procedure has been developed for the rapid and simultaneous estimation of ascorbic acid (AA) and dehydroascorbic acid (DHAA) in fresh fruits and vegetables. Isocratic separation of these components was accomplished by anion-exchange chromatography using acetonitrile–0.05 M KH_2PO_4 (75:25, v/v) as eluant. AA was determined by monitoring its absorbance at 254 nm, while DHAA detection was achieved by fluorescence as a result of postcolumn derivatization involving the condensation of DHAA with *o*-phenylenediamine (OPDA), to form a highly fluorescent quinoxaline derivative. The procedure allows detection for both forms of vitamin C at levels well below those usually found in orange juice.

INTRODUCTION

Fruits and vegetables constitute the major sources of vitamin C for human diets. The total vitamin C consists of the sum of ascorbic acid and its oxidized form, dehydroascorbic acid. Both forms have equal antiscorbutic activity (Tannenbaum, 1974).

Numerous methods for the analysis of vitamin C activity have been described. The most commonly used are the 2,6-dichlorophenolindophenol visual titration (AOAC, 1975), the spectrophotometric method with dinitrophenylhydrazine derivitization of DHAA (Roe et al., 1948), and the microfluormetric method by condensation of DHAA with OPDA (AOAC, 1975). However, these methods are not specific and are often limited by the number of interferring substances present in foods. In addition, it is difficult to visually determine the end point when these methods are used with colored solutions. Pelletier and Brassard (1977) described an improved photometric method based on 2,4-dinitrophenylhydrazine for the AA and DHAA determination in foods. Though their method eliminated interference from other compounds, it is time consuming and requires special sample preparation.

Recently, due to the development of commercial HPLC systems, quantitative measurement of AA and DHAA in various substances has been reported by many investigators. Procedures vary in the type of column, elution conditions, detection systems, and the extraction technique

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