Fluorometric Determination of Sulfite in Wine by *N*-(9-Acridinyl)maleimide

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A highly sensitive fluorometric method for determining sulfite in wine is reported. *N*-(9-Acridinyl)maleimide (NAM) reacted with the sulfite in wine quantitatively and gave strong fluorescent derivatives. The determinations were conducted by batch and high-performance liquid chromatographic methods. Sulfite contents in wine by NAM methods and the conventional methods showed good accordance. The sample size could be reduced to less than 1/10,000 by the NAM methods.

Sulfurous acid, or sulfite, is one of the common oxidized species of sulfur. In food, it is used as a food additive for antioxidation, bleaching and preservation. Especially in wine brewing, relatively large amount of sulfite are used for sterilization and as an antibacterial agent.¹⁾ It is necessary to check the level of the sulfite because of its toxicity.^{2,3)} For this determination, the Rankine⁴⁾ or its modified methods^{5,6)} have been widely used as the official methods for wine and other foods, although they are not satisfactory for sensitivity, simplicity and rapidly.

Recently, we reported a fluorometric determination of sulfite with *N*-(9-acridinyl)-maleimide (NAM) and showed several applications to determine trace amounts of sulfite in aqueous solutions.⁷⁻⁹⁾ In this paper, we report the determinations of sulfite in wine with NAM by batch and high-performance liquid chromatographic (HPLC) methods.

Materials and Methods

Materials. Sodium bisulfite ($\text{Na}_2\text{S}_2\text{O}_3$, 67.85 mg; Sigma Chem. Co., St. Louis, Mo, U.S.A.) was filled up to 50 ml with a degassed 0.02 M ethylenediaminetetraacetic acid bisodium salt ($\text{EDTA} \cdot 2\text{Na}$) solution. This 14.28 mM sulfite solution was used as the stock solution for subsequent

dilution. *N*-(9-Acridinyl)maleimide (NAM, Kanto Chem. Co., Tokyo, Japan) solutions were freshly prepared as acetone solutions just before use. Sample wines were purchased from the wine market and analyzed after diluting to 1/50 or 1/100 with a 0.02 M $\text{EDTA} \cdot 2\text{Na}$ solution. The other reagents used were super-pure or analytical grade purchased from Wako Pure Chem. Co. (Osaka, Japan).

Reaction buffer A, pH 8.8. 18.5 g of boric acid, 22.4 g of potassium chloride and 7.4 g of $\text{EDTA} \cdot 2\text{Na}$ were dissolved in 1 l of water (solution I). 31.8 g of sodium carbonate and 7.4 g of $\text{EDTA} \cdot 2\text{Na}$ were dissolved in 1 l of water (solution II). The pH value was adjusted to 8.8 by adding solution II to solution I.

Reaction buffer B, pH 10. 30.9 g of boric acid, 37.28 g of potassium chloride and 7.4 g of $\text{EDTA} \cdot 2\text{Na}$ were dissolved in 1 l of water (solution III). 53.0 g of sodium carbonate and 7.4 g of $\text{EDTA} \cdot 2\text{Na}$ were dissolved in 1 l of water (solution IV). The pH value was adjusted to 10 by adding solution IV to solution III.

Apparatus. The fluorescence spectrophotometer and isothermal incubator used were a Hitachi 204 (Tokyo, Japan) and Toyo TC-1 (Toyo Sci. Ind. Co., Tokyo, Japan), respectively. The high-performance liquid chromatograph was a Japan Spectroscopic Co., Ltd. (JASCO, Tokyo, Japan) model FLC-100 equipped with a loop type of sample injector (JASCO model VL-611). Stainless steel columns were 4.6 mm i.d. $\times$ 50 mm (PELL ODS, 30–28 μm , Whatman Co., Kent, U.K., for the precolumn) and 4.6 mm i.d. $\times$ 250 mm (JASCO SC-02, 10 μm , for the

analytical column). The fluorescence detector was a Japan Spectroscopic Co., Ltd. model FP-110 equipped with a JASCO PS-110C Hg lamp power supply.

Determination procedure—by the batch method. One ml of a sample solution (1/50 or 1/100 dilution) was added to 3.0 ml of reaction buffer A, before adding the NAM acetone solution (0.4 ml: 75 μ M). The mixture was incubated at 35°C for 2 hr and then cooled to room temperature. Its fluorescence intensity at 436 nm (excitation at 362 nm) was measured.

Determination procedure—by the high-performance liquid chromatographic method. A 50 μ l sample solution (wine diluted to 1/100) was added to 150 μ l of reaction buffer B, before adding 50 μ l of the NAM acetone solution (3.65 mM). The mixture was incubated at 50°C for more than 30 min, before a 10 μ l aliquot was injected into the HPLC. Separation was performed on an ODS column eluted with 25% acetonitrile–0.1 M ammonium acetate at a flow rate of 1.0 ml/min and monitored by fluorometry at 455 nm (excitation at 365 nm).

Modified Rankine method and the enzymatic method. To compare with the NAM methods, the total sulfite in wine was determined by the modified Rankine method and by the enzymatic method for each sample. The Rankine method was according to the procedure modified by S. Ogawa *et al.*,⁵⁾ and an F-kit sulfite (enzymatic food analysis kit for sulfite, Boehringer Mannheim Yamanouchi, Tokyo, Japan) was used for the enzymatic determination.

Results and Discussion

The reaction between sulfite and NAM has

Fig. 1. Reaction Time Course for Sulfite with NAM. Reactions were performed at 35°C and at pH 7.0 (▲), 8.8 (●) and 10.0 (■).

been previously reported.⁸⁾ This reaction proceeds in two steps, the first being the addition of sulfite to the maleimide ring of NAM, which is followed by the second step of ring hydrolysis. Both the adduct and its hydrolyzed products have strong fluorescence, and higher pH and temperature accelerate these reactions.

Figure 1 shows the fluorescence development of a reaction mixture at 35°C. Although a higher pH and temperature accelerated the reaction, the background fluorescence also increased and the net intensities of NAM-sulfite

Fig. 2. HPLC Chromatogram of Sulfite in Wine. A, NAM-sulfite adducts; B, NAM degradation products.

Fig. 3. Reaction Time Course for Sulfite with NAM. Reactions were performed at 50°C and pH 10.0. ○, sulfite standard solution; ●, white wine; ■, rose wine; ▲, red wine.

adducts decreased. At pH 8.8, the strongest intensity was obtained, and the reaction needed about 2 hr to be completed. Consequently, the reactions were performed at 35°C in pH 8.8 buffer for 2 hr by the batch method.

With the HPLC method, the background fluorescence gave almost no problem for the determination, because the peak of NAM-sulfite adducts was separated from those of NAM degradation products (Fig. 2). This made it possible to carry out the reaction at higher pH and temperature values to save the reaction time. Figure 3 shows the time course for NAM-sulfite adduct formation, the reactions being performed at 50°C in pH 10 buffer. The peak height of the adducts was almost constant after 10 min with the reaction using a standard sulfite solution. In wine, the reactions were somewhat slower and required 20 min to complete, which might be explained by the lower reactivity of the combined type of sulfite in wine. It is speculated that the combined type would become free sulfite to react with NAM. The reaction was performed at 50°C in pH 10 buffer for 30 min by the HPLC method. The calibration curves were linear in a range of sulfite concentrations from 3 to 56 μM by the batch method, and from 0.5 to 500 μM by the HPLC method. Therefore, it was necessary to dilute the wine to 1/50–1/100, because the total sulfite levels in wine are from 0.8 to 4 mM.

The sulfite contents of wine determined by the NAM, Rankine and enzymatic methods are compiled in Table I. Thirteen samples of wine were analyzed, 4 of white, 5 of red and 4 of rose wine. Their sulfite levels were 1.00–3.50 mM (about 65–225 ppm). By the NAM batch and HPLC methods, the recoveries of sulfite were $98.3 \pm 7.8\%$ and $102.4 \pm 3.8\%$ (average $\pm$ SD.) and their coefficients of variation were 2.4% ($n=8$) and 2.8% ($n=5$), respectively. Table II shows the coefficient of correlation between the methods. Although the NAM methods gave slightly higher values than those by the Rankine method (about 7% by the batch method and 10% by the HPLC method), the two NAM methods

Table I. SULFITE IN WINE

Sample	NAM method (mM)		Rankine (mM)	Enzyme (mM)
	Batch	HPLC		
White	2.28	2.28	2.12	2.23
White	3.20	3.17	2.96	2.64
White	3.17	3.15	2.90	2.62
White	2.99	3.26	2.85	2.60
Red	1.27	1.46	1.42	1.29
Red	1.86	1.99	1.95	2.03
Red	2.12	2.11	1.82	2.06
Red	1.72	1.94	1.79	2.04
Red	2.57	2.56	2.31	2.37
Rose	1.41	1.47	1.47	1.25
Rose	3.29	3.47	3.06	2.72
Rose	1.42	1.36	1.19	1.21
Rose	2.39	2.77	2.33	2.68

Table II. CORRELATION CONSTANTS BETWEEN METHODS

Method	NAM-Batch	NAM-HPLC	Rankine	Enzyme
NAM-Batch	—	0.982	0.982	0.916
NAM-HPLC	0.982	—	0.989	0.945
Rankine	0.982	0.989	—	0.928
Enzyme	0.916	0.945	0.928	—

showed good accordance with the total sulfite by the Rankine method. This implies that the NAM methods gave the total sulfite in wine, although it is not clear why the NAM methods gave higher values than the Rankine method. *N*-(9-Acridinyl)maleimide also reacts with thiols, although this should have had almost no influence because their contents in the wine were negligibly small compared to that of sulfite, and NAM-thiol adducts were separated from NAM-sulfite adducts by HPLC. The Rankine method might produce a negative error from a loss of sulfite by oxidation during handling, incomplete distillation and trapping of the sulfite. The enzymatic method showed a slightly lower value than the NAM methods, especially in wines with a relatively higher sulfite concentration. The lower values by the enzymatic method might be explained by the lower reactivity to the combined-formed sulfite at pH 8.

The NAM methods were extremely simple and sensitive, and required a sample size of less than 100 μ l. The batch method required 2 hr to complete the labeling reaction, although the simple procedures allowed us to run several samples at the same time. With the HPLC method, the labeling reaction was completed within 30 min, and HPLC determination was performed within 10 min.

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References

- 1) K. Ohtsuka, A. Totsuka, M. Ito, K. Miyazaki, K. Kuno, H. Zenibayashi, M. Kawatsu and H. Mukoyama, *J. Soc. Brew.*, **68**, 460 (1973).
- 2) M. T. Lanteaume, P. Ramel, P. Jaumles and D. Manin, *Ann. Eals. Exp. Chim.*, **669**, 231 (1969).
- 3) FAO/WHO, Evaluation of the Toxicity of a Number of Antimicrobials and Antioxidants, 6th Report, 1969, p. 12.
- 4) B. C. Rankine, *Aust. Wine Brew. and Spirit Rev.*, **80**, 14 (1962).
- 5) S. Ogawa, H. Suzuki, M. Toyoda, Y. Ito, M. Iwaida, H. Nonogi, T. Fuke, K. Obata, T. Adachi, K. Ikuzawa, T. Izumi, T. Hamano, Y. Mitsuhashi and Y. Matsuki, *Z. Lebensm. Unters. Forsch.*, **168**, 293 (1979).
- 6) F. Miyamoto and M. Saeki, *Nippon Shokuhin Kogyo Gakkaishi*, **31**, 685 (1984).
- 7) H. Meguro, C. Takahashi, S. Matsui and H. Ohruai, *Anal. Lett.*, **16A**, 1625 (1983).
- 8) K. Akasaka, T. Suzuki, H. Ohruai and H. Meguro, *Agric. Biol. Chem.*, **50**, 1139 (1986).
- 9) K. Akasaka, T. Suzuki, H. Ohruai and H. Meguro, *Anal. Sci.*, **2**, 443 (1986).