# Analysis of Diacetyl in Wine Using Solid-Phase Microextraction Combined with Gas Chromatography−Mass Spectrometry

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Analytical difficulties in the rapid and accurate determination of diacetyl (DA), an important flavor compound in wine, at low concentrations have been overcome by the use of solid-phase microextraction (SPME) with deuterated diacetyl-$d_6$ (d6-DA) as an internal standard followed by gas chromatography−mass spectrometry (GC-MS). The GC-MS analyses showed that the values of the ion response ratio of DA to d6-DA were consistent regardless of the conditions of SPME headspace and were not influenced by the presence of sulfur dioxide in wine. The quantitation value of DA was represented as the concentration of free plus bound with sulfur dioxide forms of DA. The detection limit of DA in wine was as low as 0.01 $\mu$g/mL with linearity through to 10 $\mu$g/mL.

**Keywords:** *Diacetyl; wine; flavor analysis; solid-phase microextraction (SPME); gas chromatography−mass spectrometry (GC-MS); sulfur dioxide*

## INTRODUCTION

One of the important flavor compounds in dairy products (van Neil et al., 1929; Collins, 1972) and wine (Rankine et al., 1969; Davis et al., 1985; Martineau et al., 1995) is DA, 2,3-butanedione. DA is most identifiable in wine by its buttery aroma and adds complexity to the final sensory impact of the wine. The presence of DA in wines is usually associated with malolactic fermentation (MLF), a process which can occur simultaneously with or post alcoholic fermentation. Several species of lactic acid bacteria (LAB) are able to conduct MLF. The favored species, however, is *Oenococcus oeni* (formerly *Leuconostoc oenos*, Dicks et al., 1995). Malolactic fermentation involves the decarboxylation of the dicarboxylic L-malic acid to the monocarboxylic L-lactic acid which results in a softening of the wine, due to an increase in pH, and provides microbial stability to the wine. Associated with MLF are favorable flavor modifications, such as the production of DA. DA can also be synthesized by yeast during alcoholic fermentation, albeit in small concentrations and so does not make a major contribution to the final DA content of the wine (Romano and Suzzi, 1996).

Even though DA is an important flavor compound in wine, it has not been studied and measured extensively in the past because of analytical difficulties in the quantitation caused by its highly volatile nature, low concentrations in wine, and interference of other compounds which are found in wine (Rankine et al., 1969; Laurent et al., 1994). Several different methods have been devised to determine the concentrations of DA in wine, but these techniques involve the distillation, solvent extraction, and/or derivatization of DA prior to its measurement. Colorimetric methods to measure DA have been widely used in the past, including the Prill and Hammer method (Guyman and Crowell, 1965), the Voges−Proskauer method (Walsh and Cogan, 1974), and a spectrophotometric determination method of total vicinal diketones (Garcia-Villanova and Estepa, 1993). These methods involve steam distillation to isolate DA from the wine matrix. However, distillation has the disadvantage of an incomplete fractionation of DA from the closely related compounds, α-acetolactate, a precursor to DA, and acetoin which will result in an overestimation of the DA concentration in wine. A fluorometric method was developed by Mariaud and Levillain (1994) to improve upon the lengthy distillation methods which involves the reaction of DA with 2,3-diaminonaphthalene to yield the 2,3-dimethyl[2,3-*b*]naphthopyrazine which has fluorescent properties. Another method is to derivatize DA with 4,5-dichloro-1,2-diaminobenzene to yield the 6,7-dichloro-2,3-dimethylquinoxaline complex, and its concentration is determined by gas chromatography, combined with mass spectrometry (Martineau et al., 1994) or electron-capture detection (Otsuka and Ohmori, 1992). These chromatographic methods have excellent specificity and sensitivity, although accuracy in measured concentrations of DA in wine may be doubtful due to the severe derivatization procedure.

SPME is a fast, simple, and solventless alternative sampling technique (Arthur and Pawliszyn, 1990; Zhang and Pawliszyn, 1993) and has been applied to a number of flavor and taint analyses of beverages (Garcia et al., 1996; Yang and Peppard, 1994; Hawthorne et al., 1992; Fischer and Fischer, 1997). In particular, headspace SPME would be the favored choice for the analysis of DA in wine because of the high volatility of the analyte, and also the headspace method reduces any interferences from other wine constituents in wine. Combined with GC-MS, SPME should be an excellent method for selectively identifying and quantifying DA in wine.

The accuracy and precision of quantitative analysis in mass spectrometry is strongly related to the choice of an internal standard. Ideally, the sources of the variation on an analytical procedure should affect both the sample and internal standard in the same manner. For this reason the stable isotope labeled analogue of an analyte is the most suitable form of the internal standard (Chapman, 1993).

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In this paper we describe the development of an accurate method for the determination of DA in wine using headspace SPME combined with GC-MS. This method involves minimal sample preparation and utilizes a deuterated form of DA as the internal standard. Since the aroma sensory threshold for DA in wine is more than 0.2 μg/mL (Martineau et al., 1995), a detection limit was targeted at 0.01 μg/mL of DA in wine to allow for a comparative study of DA concentration and sensory impact.

## MATERIALS AND METHODS

**DA and SPME.** DA was purchased from Sigma (St. Louis, MO), and d6-DA used as an internal standard was purchased from CDN Isotopes (Quebec, Canada). The SPME holder for manual sampling and the fiber coated with a 100 μm poly(dimethylsiloxane) and a 65 μm Carbowax−divinylbenzene were purchased from Supelco (Bellefonte, PA).

**Sample Preparation and Headspace SPME Procedures.** Commercial Australian white (Chardonnay) and red (Shiraz and Cabernet Sauvignon) wines containing 12%−14% of alcohol were used, or synthetic wine (11%) was prepared according to Liu et al. (1994) and used for the calibration curve of DA. Headspace SPME was applied for all analyses. The SPME fiber was exposed to the headspace above 3 mL of the wine sample in a 15 mL glass vial with a Teflon-coated septum. Effects of the addition of salt (sodium chloride) to wine, SPME fiber coating, temperature, and length of sampling on the analysis of DA were investigated. Immediately after completion of the SPME step, the analytes absorbed in the SPME fiber were analyzed by GC-MS.

**GC-MS Analysis.** The GC-MS analysis was carried out using a Varian model 3400 gas chromatograph combined with a Finnigan Mat TSQ 70 mass spectrometer. Analytes were thermally desorbed from the coated fiber of the SPME in the hot injector of the GC and were separated on a 50 m × 0.32 mm i.d. fused silica capillary column of Chrompack WCOT FS CP-Wax 57 CB with a 1.2 μm film thickness. The GC column was maintained at 60 °C for 1 min, ramped at a rate of 5 °C/min to 110 °C, and then further ramped at a rate of 20 °C/min to 180 °C and held at this temperature for 10 min. The fiber remained in the injector until completion of analysis in order to condition it for the next analysis. The split−splitless injector was used, and the split vent was opened after 0.5 min. Temperatures of the injector and the transfer line were 200 and 220 °C, respectively. The carrier gas was helium with a column head pressure of 14 psi at 60 °C. The molecular ions of DA (*m*/*z* 86) and of the d6-DA (*m*/*z* 92) were scanned in selected ion monitoring mode with 0.2 s of a dwell time under the mass spectrometric conditions as follows: electron impact with 70 eV, 150 °C of the ion source temperature, and 200 μA of emission current. The concentration of DA in wine was determined by the ratio of the ion response of DA relative to that of the d6-DA.

## RESULTS AND DISCUSSION

**Development of the Method.** It has been reported that the extraction efficiency of the SPME method is dependent upon the choice of the SPME fiber coating, length and temperature of sampling, the addition of salt to the sample and stirring of the sample (Garcia et al., 1996). A 60 μm Carbowax−divinylbenzene (CD) coating fiber was chosen because its sensitivity was more than five times greater than that of a 100 μm poly(dimethylsiloxane) when 0.1 μg/mL of DA in water (3 mL) was extracted for 10 min at 40 °C (data not shown). This is thought to be due to the high polarity of DA where polar analytes are expected to exhibit a larger value of distribution constant in a more polar stationary phase than in a less polar one. The CD coating fiber was used in all subsequent experiments.

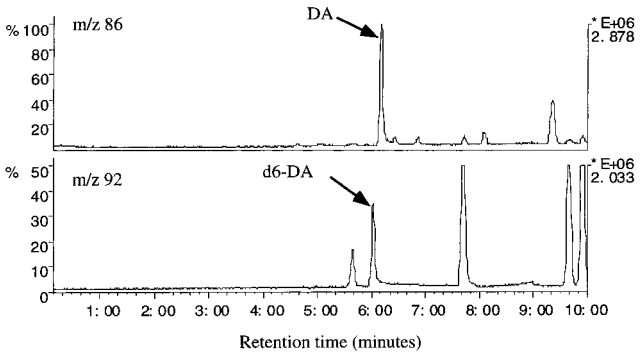


**Figure 1.** Ion chromatograms of *m*/*z* 86 for diacetyl (DA) and of *m*/*z* 92 for $d_6$-deuterated diacetyl (d6-DA) extracted from red wine with 1.5 g of salt added.

For the quantitation of DA in wine, d6-DA was used as an internal standard, and its stability, as the deuterated form, in wine matrixes was examined by two methods. First, the values of the ion response ratio of *m*/*z* 90 (d4-DA) and *m*/*z* 91 (d5-DA) to *m*/*z* 92 of d6-DA were obtained at 0 and 30 min after the addition of d6-DA (100 μg/mL) to both red and white wines. Headspace SPME was carried out for 5 min at 24 °C with the addition of 1.5 g of sodium chloride, followed by GC-MS analysis in scan mode with a mass range from *m*/*z* 80 to 100 with 0.5 s of a scan time. The values of the ratio of *m*/*z* 91 to 92 were 7.3% in red and 7.2% in white wine at 0 time and 7.4% and 7.2% at 30 min, respectively, and the values of the ratio of *m*/*z* 90 to 92 were less than 0.5% at all the samplings. The values of the ratio of either *m*/*z* 90 or 91 to 92 of d6-DA remained almost constant in 30 min. Second, the values of the ion response ratio of naturally occurring DA to d6-DA (1 μg/mL) in red and white wines were determined at 0, 1.5, and 4.5 h after the addition of d6-DA. Headspace SPME was carried out, as described above, followed by GC-MS in selected ion mode (*m*/*z* 86 for DA and 92 for d6-DA). The values of the ion response ratio over the 4.5 h increased only 1.1% in red and 6.5% in white wine. The results from both experiments indicate that the substitution of deuterium of d6-DA with hydrogen in wine matrixes appears to be very slow. In practice, the length of SPME sampling after the addition of the internal standard is usually less than 15 min; therefore, d6-DA is sufficiently stable to act as an internal standard.

Headspace SPME conditions were examined using 3 mL samples of commercial Australian red wines containing naturally occurring DA and with the addition of 3 μg of d6-DA (final concentration: 1 μg/mL). The vial was vigorously shaken for 10 s prior to the commencement of headspace SPME. The effect of the addition of salt (sodium chloride) on the extraction of DA was examined by the addition of varying concentrations of salt (0.5−2 g) to wine prior to the headspace SPME for 3 min at 30 °C. Figure 1 shows the ion chromatograms of *m*/*z* 86 for DA and of *m*/*z* 92 for d6-DA (1 μg/mL) extracted from the red wine with 1.5 g of salt added. The peak of d6-DA appeared at a retention time of 6 min, 8 s earlier than that of DA. Both the peaks were clearly free from interference by other GC eluents. Figure 2 demonstrates that the ion response of DA detected by GC-MS increased with an increase in salt addition to the wine. The increases of the ion responses

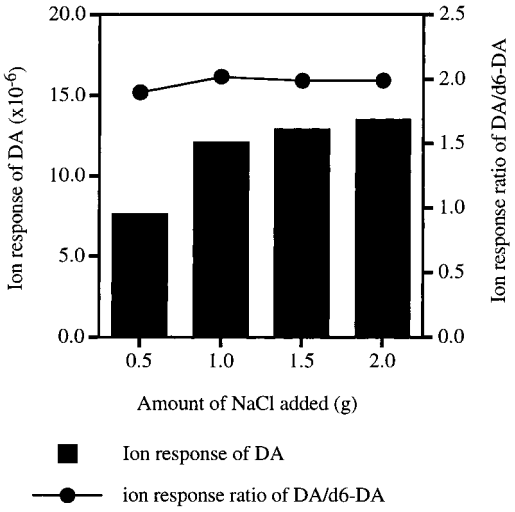


**Figure 2.** Effect of the addition of salt (NaCl) on the extraction of diacetyl (DA) from red wine.

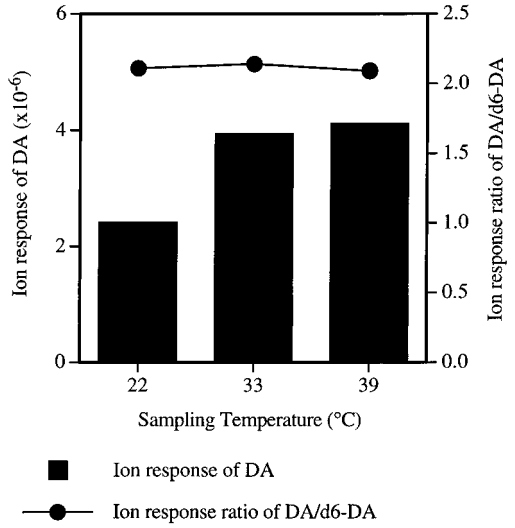


**Figure 3.** Effect of temperature on the extraction of diacetyl (DA) from red wine.

of 1.0, 1.5, and 2.0 g as compared to 0.5 g of salt addition were approximately 1.6, 1.7, and 1.8-fold, respectively, indicating that the salting-out effect obviously took place. The addition of 1.5 and 2.0 g of salt slightly enhanced the value of the ion response of DA as compared to 1.0 g of salt addition, suggesting that the salting-out effect reached saturation above 1.0 g of salt addition. The values of the ion response ratio of DA to d6-DA over the range of salt additions are shown in Figure 2 and are relatively consistent, with an average of 1.97 and a coefficient of variation (CV) of 2.5%. This observation demonstrates that the values of the ion response ratio are constant regardless of the significant salting-out effect.

The effect of temperature on the extraction of DA from the wine was examined over a range of 22−40 °C. Headspace SPME was carried out for 3 min with the addition of 1.5 g of sodium chloride. Figure 3 shows that the temperature at which the extraction of DA is carried out does influence the extraction of DA. The ion response of DA increased 65% at 33 °C from 22 °C, but it only increased slightly (5%) from 33−39 °C while the values of the ion response ratio of DA to d6-DA remained constant throughout all temperatures with an average of 2.11 (CV = 1.5%).

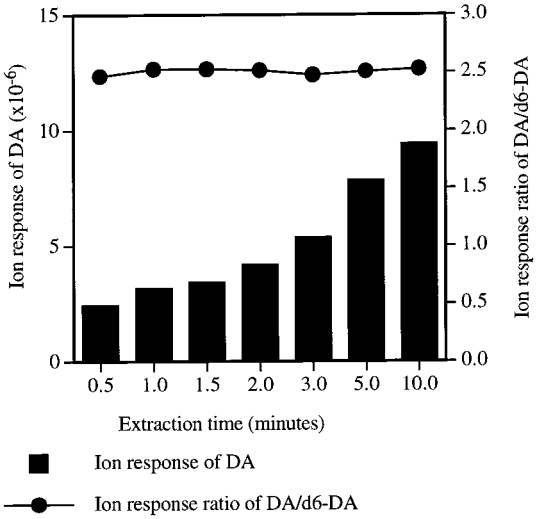


**Figure 4.** Effect of the length of the sampling time on the extraction of diacetyl (DA) in red wine.

The effect of the length of the sampling time on the extraction of DA from the wine is shown in Figure 4. Headspace SPME was carried out at 30 °C with an addition of 1.5 g of sodium chloride. The ion response of DA increased linearly over a range of 0.5−5 min and then only slightly up to 10 min, while the values of the ion response ratio of DA to d6-DA remained constant throughout all extraction times with an average of 2.51 (CV = 0.9%).

We have demonstrated that the ion response of DA, which is directly related to the sensitivity of the analysis, can be improved and optimized by changes in the addition of salt to the wine and temperature and length of DA sampling, while the ion response ratio of DA to d6-DA remains constant regardless of the changes in any of the above conditions. The procedure devised for the practical extraction of DA from wine by headspace SPME utilizes 3 mL of wine with the addition of d6-DA (final concentration: 1 $\mu$g/mL) and 1.5 g of sodium chloride, shaken manually for 10 s followed by headspace SPME using the CD coating fiber for 3 min at ambient temperature.

The ion response ratio of DA to d6-DA with known concentrations of DA was determined using the procedure devised (as described above) to examine the linearity between DA concentration and the ion response ratio (DA/d6-DA). A calibration curve throughout a range of DA concentration from 0.01 $\mu$g/mL to 10 $\mu$g/mL in the synthetic wine showed a high linearity over the entire range having a correlation coefficient greater than 0.999. The synthetic wine sample with no addition of DA (blank) showed the flat baseline where DA was expected to emerge. The detection limit of DA in the synthetic wine was estimated to be less than 0.01 $\mu$g/mL, as the net signal of this concentration was approximately 7 times greater than the standard deviation ($n$ = 4) of the baseline signals close to both sides of the actual DA peak. The sampling time for the signals of both DA peak and the baseline was taken for 9 s in duration.

**Method Validation.** The repeatability of the method was investigated by repeating the headspace SPME analysis (6 times on the same day) of the same wine sample. The results are shown in Table 1. The method was highly repeatable in both the red and white wines, as a coefficient of variation of the DA concentration in

**Table 1. Repeatability of the Quantitation Values of Diacetyl (DA) in White and Red Wines Obtained by the Headspace SPME Method**

| | ion response ($\times 10^{-6}$) | | |
|---|---|---|---|
| anal. no. | DA | d6-DA | ratio DA/d6-DA |
| | Red Wine | | |
| 1 | 25.04 | 11.79 | 2.124 |
| 2 | 27.74 | 13.40 | 2.070 |
| 3 | 28.86 | 13.93 | 2.072 |
| 4 | 26.95 | 13.07 | 2.062 |
| 5 | 27.77 | 13.24 | 2.097 |
| 6 | 25.13 | 12.02 | 2.091 |
| | | | 2.086 (av) |
| | | | 1.1 (CV (%)) |
| | White Wine | | |
| 1 | 6.63 | 36.20 | 0.183 |
| 2 | 7.20 | 38.94 | 0.185 |
| 3 | 6.28 | 33.57 | 0.187 |
| 4 | 6.07 | 33.02 | 0.184 |
| 5 | 7.42 | 37.87 | 0.196 |
| 6 | 7.08 | 38.44 | 0.184 |
| | | | 0.187 (av) |
| | | | 2.6 (CV (%)) |

**Table 2. Recovery Test of the Spiked Diacetyl (DA) in Red and White Wines**

| | concentration of DA ($\mu$g/mL) | | | | |
|---|---|---|---|---|---|
| wine no. | present | spiked | expected | obsd | recovery (%) |
| | | White Wine | | | |
| 1 | 0.045 | 0.500 | 0.545 | 0.512 | 93.9 |
| 2 | 0.052 | 0.500 | 0.552 | 0.570 | 103.3 |
| 3 | 0.051 | 0.500 | 0.551 | 0.550 | 99.8 |
| 4 | 0.049 | 0.500 | 0.549 | 0.581 | 105.8 |
| 5 | 0.107 | 1.000 | 1.107 | 1.075 | 97.1 |
| | | | | | 100.0 (av) |
| | | | | | 4.7 (CV (%)) |
| | | Red Wine | | | |
| 1 | 1.520 | 1.000 | 2.520 | 2.360 | 93.7 |
| 2 | 1.500 | 1.000 | 2.500 | 2.495 | 99.8 |
| 3 | 1.478 | 1.000 | 2.478 | 2.498 | 100.8 |
| 4 | 2.722 | 1.000 | 3.722 | 3.716 | 99.8 |
| 5 | 0.173 | 0.500 | 0.673 | 0.670 | 99.6 |
| | | | | | 98.7 (av) |
| | | | | | 2.9 (CV (%)) |

the red and white wine was 1.10% and 2.58%, respectively. In addition, the values of the ion response of DA and d6-DA obtained by the repeated analyses were relatively consistent, having a coefficient of variation of less than 8% in either the red or white wine. The ion response of d6-DA from the white wine sample was approximately three times higher than that from the red wine; we attribute this due to a difference in matrix effect such as the levels of alcohol and sulfur dioxide. The results indicate that the concentration of DA obtained by the headspace SPME method is highly repeatible in either red or white wine.

The accuracy of the method in measuring DA concentration of wine was investigated by conducting a DA recovery test. The test was performed by measuring naturally occurring DA in the wine followed by measuring the same wine spiked with a known concentration of DA (0.5 or 1 $\mu$g/mL). Recovery of DA was determined by comparing the observed and expected total concentrations of DA (original DA content plus DA spiked) in the wine, and the results are shown in Table 2. Recoveries of DA in white and red wines exhibited averages of 100.0% and 98.7% with a coefficient of variation of 4.7% and 2.9%, respectively. The result indicates that the concentration of DA in either red or white wine is accurately determined by the headspace SPME method.

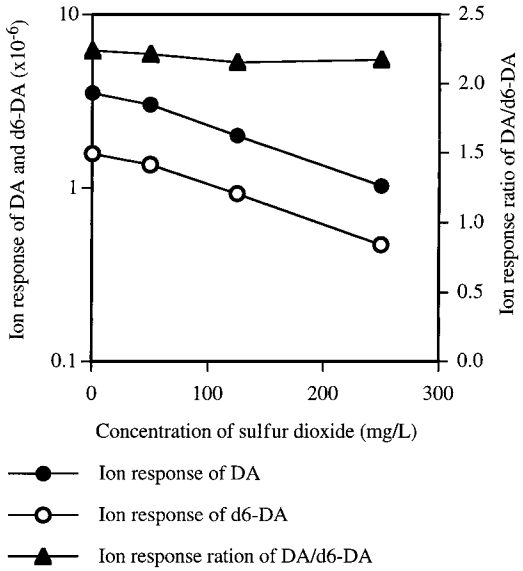


**Figure 5.** Degree of the binding of diacetyl (DA) with sulfur dioxide in the synthetic wine.

**Effect of Sulfur Dioxide.** DA is thought to reversibly bind with sulfur dioxide in wine (Nielson and Prahl, 1995). It was of interest to determine whether the developed method was measuring DA as a free form or as a free plus sulfur dioxide bound form in wine. To investigate the effect of sulfur dioxide on DA measurement, two experiments were carried out. The first experiment was to investigate the degree of the binding of DA to sulfur dioxide in the synthetic wine and its consequent effect on the quantitation. After the addition of 3 $\mu$g of DA to 3 mL of the synthetic wine containing potassium metabisulfite equivalent to sulfur dioxide concentrations of 0, 50, 125, or 250 mg/L, the synthetic wine was left for 5 min at ambient room temperature and then the values of the ion response of DA and d6-DA were measured according to the method described above. Figure 5 illustrates that the ion response of DA decreases exponentially as the concentration of sulfur dioxide increases, while the values of the ion response ratio of DA to d6-DA throughout a range from 0 to 250 mg/L added sulfur dioxide are consistent with an average of 2.19 (CV = 1.7%). The degree of the binding of DA or d6-DA with sulfur dioxide was estimated by comparing the value of the ion response from the synthetic wine containing sulfur dioxide to that from the sample with no sulfur dioxide. It exhibited 14.5% of the binding of DA with sulfur dioxide at 50 mg/L of sulfur dioxide, 43.4% at 125 mg/L, and 71.0% at 250 mg/L while that of d6-DA was 13.5%, 41.2%, and 70.1%, respectively. The binding of DA and d6-DA to 1 mg/L of sulfur dioxide was calculated to be 0.29% and 0.27% at 50 mg/L of sulfur dioxide, 0.34% and 0.33% at 125 mg/L, and 0.28% and 0.28% at 250 mg/L, respectively. These values of DA showed good agreement with those of d6-DA and were relatively consistent (CV= 10%) regardless of the concentration of sulfur dioxide. It indicates that DA and d6-DA react in similar way in the presence of sulfur dioxide; therefore, the addition of d6-DA allows to compensate to obtain the constant values of the ion response ratio regardless of the concentrations of sulfur oxide.

The second experiment was to investigate a rate of the binding of naturally occurring DA to sulfur dioxide

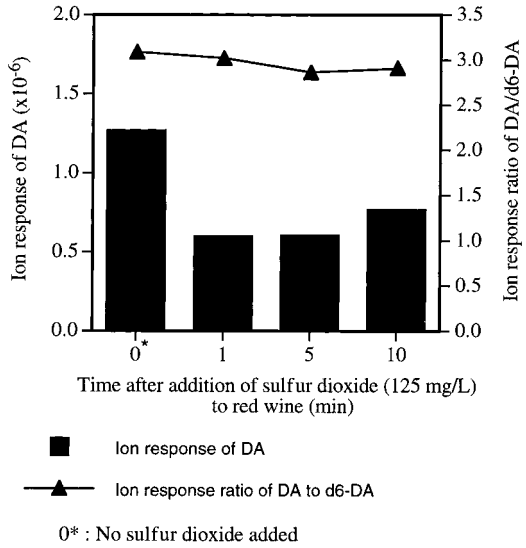


**Figure 6.** Rate of the binding of diacetyl (DA) with sulfur dioxide in red wine.

in red wine and its consequent effect on the quantitation. DA in the red wines was measured at 0 min with no sulfur dioxide added and at 1, 5, and 10 min after the addition of 125 mg/L (final concentration in the wines) of sulfur dioxide. All samples were subjected to headspace SPME after the addition of d6-DA and salt. Figure 6 shows that the values of the ion response of DA decrease significantly after the addition of sulfur dioxide, while the values of the ion response ratio over all sampling times including 0 min are consistent with an average of 2.94 (CV = 3.39%). The value of the ion response of DA at 1 min after sulfur dioxide addition was approximately 50% as compared to that at 0 min and maintained a similar level thereafter. The different measurement times after the addition of sulfur dioxide did not change significantly the values of the ion response ratio.

The headspace SPME technique is thought to extract only the free forms of DA and d6-DA from wine. If the binding reaction of DA and d6-DA with sulfur dioxide is slow, the method would determine only the concentration of the free form of DA in wine, but if the reaction is rapid, it would be possible to determine the total DA concentration (the free plus bound forms). Both experiments showed that the values of the ion response of DA and d6-DA decreased considerably in the short periods of time after the addition of sulfur dioxide, indicating that the rate of the binding reaction was rapid. The first experiment showed that the ion response ratio of DA to d6-DA, which is directly related to the quantitation value of DA, was consistent regardless of the concentration of sulfur dioxide, indicating that the SPME method determined the total DA concentration in the synthetic wine. The second experiment showed that the ion response ratio of DA to d6-DA did not vary during the different sampling times after the addition of sulfur dioxide, indicating that d6-DA reacted rapidly with sulfur dioxide and reestablished an equilibrium with DA and sulfur dioxide in a very short period of time. These findings imply that the rate of the binding reaction of DA and d6-DA with sulfur dioxide is sufficiently rapid to allow the determination of the total DA concentration in wine.

## CONCLUSION

In general, the precision, accuracy, specificity, convenience, robustness, sensitivity, and applicability to a variety of matrixes are of concern during the development of an analytical method (Green, 1996). The method developed for DA using headspace SPME combined with GC-MS showed excellent precision, accuracy, and applicability in both white and red wines as verified by the repeatability and recovery tests conducted, as discussed above. The convenience criterion was satisfied with the small sample volume (3 mL) and by utilizing SPME, which is a simple, rapid, and solvent-free extraction technique. Using a deuterated form of DA (d6-DA) as an internal standard ensured the robustness of the method in that the quantitative value of DA would not be affected by changes in the parameters of the headspace SPME conditions. High specificity was ensured by using GC-MS in selected ion monitoring mode and demonstrated excellent linearity throughout a wide concentration range of DA (0.01−10.0 $\mu$g/mL). The quantitation values represented the concentration of free and bound with sulfur dioxide forms of DA. The targeted detection limit of 0.01 $\mu$g/mL was achieved, but it has been confirmed that sensitivity can be significantly improved by changes in the parameters of SPME as described above and, in addition, by means of chemical ionization mode in GC-MS (data not shown).

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