



Comparison and optimization of three commercial methods with an LC–MS/MS method for the determination of sulfites in food and beverages

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ABSTRACT

Sulfites are food additives found in a large variety of food products to help reduce oxidation and browning. A small subset of the population has “allergic-like” symptoms upon consumption of sulfite containing foods. There are several sulfite detection methods available in the literature that vary in analysis time and required instrumentation. There are also rapid screening tests available that have not been tested extensively for accuracy and precision. In this study, four different methods (an LC–MS/MS method, a spectrophotometric method, a test strip method and a spot test method) were used to determine the sulfite concentration of 10 different commercially sulfited products. Of these methods, the LC–MS/MS and the spectrophotometric method had the most comparable results. The test strips were only accurate at concentrations greater than 50 mg/kg (ppm) SO₂. The spot test method was found to be inaccurate with both standard solutions and samples.

1. Introduction

Sulfites are a family of food additives that are used in a variety of products mainly to prevent oxidation and browning. These additives are approved for use in food products in the U.S. and many other countries worldwide. However, they must be declared on the label if their residual concentration is greater than 10 mg/kg (ppm) SO₂ (U.S. Food Drug Administration, 2018). The US regulations were approved in the 1980s when it was discovered that a small subset of the population was sensitive to sulfites and experienced “allergic-like” reactions upon consumption of foods containing high concentrations of sulfites (Taylor, Higley, & Bush, 1986). The US FDA selected the AOAC Official Method 990.28 (AOAC International, 2000), the Optimized Monier-Williams Method, (OMW) as the official method for regulatory samples when the regulations were finalized.

Since that time, additional methods have been published using a variety of techniques such as high-performance liquid chromatography (HPLC) (Theisen, Hansch, Kothe, Leist, & Galensa, 2010), liquid chromatography tandem-mass spectrometry (LC–MS/MS) (Carlos & de Jager, 2017; Robbins, Shah, MacMahon, & de Jager, 2015), ion chromatography (IC) (Cooper, Marshall, Gregory, & Otwell, 1986; Iammarino et al., 2010) and electrochemistry (Isaac, Livingstone, Wain, Compton, & Davis, 2006; Montes et al., 2012). These methods show some advantages to the OMW in sensitivity, selectivity, and analysis time. However, they require expensive instrumentation and specialized

training. For this reason, several manufacturers have recently introduced more rapid methods that do not require specialized instrumentation. The targeted audience for these methods includes not only food manufacturers but also sulfite sensitive consumers. While these methods are rapid and easy to use, there is no literature data available regarding their accuracy or precision for identifying added sulfites in sulfite containing matrices. If these inexpensive methods are shown to be accurate, they could provide a useful tool to consumers, food retailers and industry.

The present study will investigate three rapid tests as well as a newly validated LC–MS/MS method in ten different matrices including dried fruits, dried vegetables, and juices. The LC–MS/MS method uses a 0.2% formaldehyde extracting solution to remove sulfite from the matrix and convert it to a stable adduct, hydroxymethylsulfonate (Carlos & de Jager, 2017; Robbins et al., 2015). This adduct can then be separated using hydrophilic interaction liquid chromatography (HILIC) and detected using tandem mass spectrometry. One of the rapid tests, the Sulfite Assay Kit, is measured spectrophotometrically and is based on two different enzyme catalyzed reactions (MaxSignal Sulfite Assay Manual, 2016). In the first redox reaction, sulfite is oxidized to sulfate and oxygen is reduced to hydrogen peroxide. Hydrogen peroxide then oxidizes NADH to NAD⁺ in the second reaction. The decrease of the NADH is measured by a spectrophotometer at 340 nm and is proportional to the sulfite concentration of the sample. The other two tests, the sulfite test strips and the Spot test, are colorimetric tests but the

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manufacturers do not include any information regarding their mechanism of action for sulfite determination. The test strips are dipped either directly into the liquid food samples or into a matrix extract. The color change of the strip can then be compared to a standard scale provided by the manufacturer. For the spot test, the sample or matrix is applied to the paper and then the spot density is measured using a densitometer. An investigation is necessary in order to determine if the accuracy and precision of these rapid tests is comparable to that which can be achieved with a highly selective and sensitive method such as the LC–MS/MS method.

2. Materials and methods

2.1. Reagents and materials

Sodium sulfite (Na_2SO_3 , $\geq 98\%$) and the internal standard sodium sulfite- ^{34}S ($\text{Na}_2^{34}\text{SO}_3$, 95%), in addition to formaldehyde (37%, stabilized with 10–15% methanol), ammonium acetate (Sigma Ultra, minimum 98%), poly(vinylpyrrolidone) (PVPP), ascorbate oxidase (from *Curcubita* sp., lyophilized powder, 1.000–3.000 units/mg protein), citric acid ($\geq 99.5\%$), and Quantofix® test strips were purchased from Sigma-Aldrich (St. Louis, MO, USA). LC–MS grade acetonitrile, water and methanol, as well as methylene chloride (99.9%), sodium hydroxide pellets (99.5%), glacial acetic acid, hydrochloric acid (36.5–38.0%), acetone (Optima, 99.6%), ethanol ($\geq 99.5\%$) and sodium acetate trihydrate were acquired from ThermoFisher Scientific (Pittsburgh, PA, USA). Sulfite Assay Kits were purchased from Bio Scientific (Austin, TX, USA). Samples were diluted and extracted using 18 M Ω water obtained from an Aqua Solutions water purification system (Jasper, GA, USA). The spot tests were acquired from the Spot Test Company (Watkinsville, GA, USA). The sulfited samples were purchased online or from grocery stores located in Greenbelt, MD, USA. All food samples were stored according to the manufacturer's suggested storage conditions.

2.2. LC–MS/MS

The sulfite concentration was determined by LC–MS/MS using the method of Carlos and de Jager (2017). Briefly, free and reversibly bound sulfite reacts with formaldehyde to form the stable adduct, hydroxymethylsulfonate. The food samples were extracted with a buffered 0.2% formaldehyde solution combined with a sodium sulfite stable isotope ($\text{Na}_2^{34}\text{SO}_3$) and injected for analysis into a UPLC coupled with a mass spectrometer. Both an Acquity (Waters, Milford, MA, USA) and a Nexera X2 (Shimadzu, Columbia, MD, USA) were used for separation by hydrophilic interaction liquid chromatography (HILIC). An AB Sciex 4000 QTRAP mass spectrometer equipped with an electrospray ionization (ESI) source in negative ion mode was used for analysis. Specific chromatographic and instrument parameters are included in the original publication.

2.3. Sulfite assay kit

Standards were prepared according to the manufacturer's protocol with slight modifications. The protocol calls for a 6-point calibration curve ranging from 0 to 300 ppm. To improve accuracy of the curve fit, we added an additional 6 points for a total of 12. The range of the calibration curve remained the same. Volumes of standard added to the cuvette (Hellma Herasil quartz, pathlength 260–2500 nm spectral range, pathlength 10 mm, chamber volume 1.4 mL; Sigma Aldrich, Milwaukee, WI) were adjusted for peak curve fit.

The manufacturers include a variety of different sample preparations in their recommended directions. Several different sample preparations had to be used due to the variety of matrices investigated. The matrices used for each of the following manufacturer's preparations included: dried fruit (apricot, coconut, ginger, mango, papaya,

Table 1

Sulfite Assay Kit sample preparation and volume for dried fruits and vegetables.

Sample	Mass of sample (g)	Extracting solvent volume (mL)	Volume of extract in cuvette (μL)
Apricot	12 ± 0.1	160 ± 1	320
Coconut	36 ± 0.1	120 ± 1	660
Ginger	16 ± 0.1	160 ± 1	660
Mango	12 ± 0.1	160 ± 1	660
Papaya	24 ± 0.1	160 ± 1	660
Pineapple	12 ± 0.1	160 ± 1	660
Potato	30 ± 0.1	160 ± 1	660
Tomato	12 ± 0.1	160 ± 1	320

pineapple, potato and tomato), starch (potato), fruit juice (white grape juice) and white wine (white wine vinegar). In the case of dried fruits and vegetables, small adjustments to the recommended extraction protocol were made to achieve more accurate results. For the dried fruits and vegetables, between 12 ± 0.1 g and 36 ± 0.1 g of the sample were chopped in 2 cm pieces and homogenized together with 120–160 mL of 18 M Ω heated water ($65 \pm 1^\circ\text{C}$) in a variable-speed laboratory blender (Waring Laboratory Science, Torrington, CT, USA) (Table 1). The homogenate (43 ± 0.1 g) was transferred into a 50 mL polypropylene centrifuge tube and put in an overhead shaker with vertical rotation (Trayster digital, IKA, Wilmington, NC, USA) at 70 rpm for 10 min. After that, the tubes were centrifuged at $4000 \times g$ for 10 min (Marathon 2100R centrifuge, model 120, Thermo Fisher Scientific). The supernatant (10 mL) was transferred into a 15 mL centrifuge tube and 0.75 ± 0.05 g of PVPP was added. The tubes were shaken and centrifuged again, all supernatant was transferred into a clean 15 mL centrifuge tube and approximately 2 mL of it was filtered through a syringe PTFE filter ($0.45 \mu\text{m}$, 17 mm, Thermo Fisher Scientific/ $0.22 \mu\text{m}$, 25 mm, Grace, Columbia, MD, USA). Different volumes of the prepared sample were used for the spectrophotometer to improve the accuracy of the assay. These volumes can be seen in Table 1. It should be noted that total volume of all reagents and dilution solvents was held constant across all measurements.

It was important that the cuvettes were cleaned properly after use to ensure accurate results. A cleaning solution containing 100 mL ethanol, 75 mL 18 M Ω water and 25 mL concentrated hydrochloric acid was used to soak each cuvette for 30 s. The cuvette was then rinsed three times with 18 M Ω water and the outside of the cuvettes wiped three times with the cleaning solution followed by three times with 18 M Ω water. Finally, the cuvettes were flushed with acetone and allowed to air dry.

Cuvette preparation and quantitation were performed following the manufacturer's instructions with no deviations. A linear fit was used for the calibration curve and all curves had R^2 values > 0.95 .

2.4. Quantofix® test strips

Since the strips are best designed for dipping into a liquid matrix, a sample preparation was developed for solid matrices. Depending on the matrix investigated, 7.5–25 g of the samples were cut into ~ 2 cm pieces and homogenized together with 70 ± 1.0 mL of 18 M Ω water in a laboratory blender. The homogenate (10 ± 0.1 g) was transferred into a 50 mL centrifuge tube, approximately 1 g of sodium acetate and 0.75 ± 0.1 g of PVPP were added to it. For the liquid samples, 10 ± 0.1 g was mixed with between 1.0 ± 0.1 g and 8.0 ± 0.1 g sodium acetate and 0.75 ± 0.1 g of PVPP in a 50 mL centrifuge tube. The sodium acetate was added per the manufacturer's instructions to buffer the solution to a pH of 6–9. Due to the wide variety of matrix pH, different amounts of sodium acetate were required. The PVPP was added to remove some of the color components of the fruit and vegetable mixtures which could lead to false positives. All tubes were vortexed for 1 min (Thermolyne Vortex Maxi Mix Plus Mixer, Barnstead

International, Dubuque, IA, USA) and centrifuged for 5 min at $4000 \times g$. The supernatant was used for analysis.

The Quantofix® test strips (Sigma Aldrich) were dipped into the supernatant for one second and the liquid was shaken off. After 20 s, the color of the test field was compared to a sulfite scale on the box of the strips. The scale ranges from 10 to 1000 mg/L SO_2 . The determined concentration was adjusted for any dilution during sample preparation to calculate the actual sulfite concentration in the sample.

2.5. Spot test strips

No directions for use were provided with the Spot Test Strips. A method was developed in our laboratory. A 1000 mg/L SO_3^{2-} stock solution was made up by dissolving 0.157 ± 0.005 g sodium sulfite in 100 mL of 18 M Ω water. It was diluted to eight calibration curve points (10, 25, 50, 100, 200, 300, 400 and 500 mg/L SO_3^{2-}) with 18 M Ω water. Each standard (200 μL) was pipetted on a blue spot test strip and the strip was allowed to air dry.

The solid samples (5.0 ± 0.1 g) were homogenized in a tube mill (Tube-Mill 100 control, IKA). After weighing 1.0 ± 0.1 g of the homogenate in a 15 mL centrifuge tube, 1.3 ± 0.3 g of 18 M Ω water was added along with 0.090 ± 0.020 g PVPP (when necessary to remove possible interfering pigments from the solutions). All tubes were centrifuged for 5 min at $4000 \times g$. The supernatant of the solid samples and the liquid samples (200 μL) were pipetted on the spot test strips and the strip was allowed to air dry.

The spot density of the spot test strips was measured by a densitometer (AlphaImager 3400, Alpha Innotech, Kasendorf, Germany) in the 2-D spot densitometry mode. The reflective white light source was used and set to auto exposure. The filter was set in Position #2 which is used for colorimetric analyses. The area of interest was outlined manually, and the auto background function was used for acquisition. The instrument calculates the integrated density value (IDV) by subtracting the background of the area of interest. All IDVs were divided by the IDV of a blank and then put in a diagram over the natural logarithm of the standard concentration.

3. Results and discussion

All four sulfite methods were used to analyze ten commercially sulfited matrices ranging from white grape juice to dried apricot. Sample preparation was optimized to ensure that each method was operating under optimal conditions based on spike recovery values of standards. The spot test method never produced a usable standard calibration curve (Fig. 1). There was no correlation that could be determined between the IDV value of a standard and the standard concentration. For this reason, no sulfite concentrations could be determined for any of the matrices studied and data for this method does not appear in any of the tables or figures of this study. Triplicate analyses from the same homogenate were performed on the other three methods. The test strip is only semi-quantitative due to the non-continuous scale of the test strips. For this reason, determining average and standard deviation was not possible. Instead, the most commonly occurring response (the mode) was chosen as the result. The most commonly occurring response was then adjusted based on the dilution factor for that matrix, converted into SO_2 and this concentration was presented (Table 2).

The LC-MS/MS method was able to determine a sulfite concentration for all the matrices studied. The relative standard deviations of the samples were quite small ranging from 2.4% in mango to 7.0% in tomato. These were much smaller than the deviations seen with the sulfite assay kit which was also able to determine the sulfite concentrations for all matrices investigated. The relative standard deviations for the sulfite assay kit ranged from only 7% with the pineapple sample to 266% in the dried coconut. The sulfite assay kit tended to have the lowest relative standard deviation for the samples that had the highest

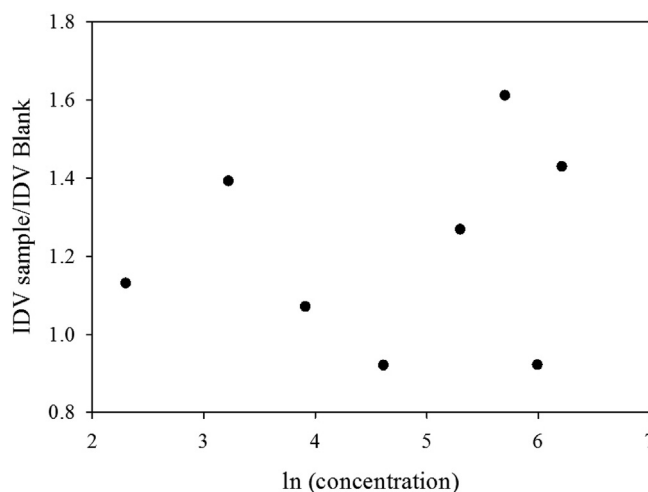


Fig. 1. Calibration points for the Spot Test presented as $\ln$ (concentration) vs background corrected IDV.

Table 2

Results of three sample methods for 10 commercially sulfited food products.

Sample Matrix	LC-MS/MS ¹	Sulfite Assay Kit ²	Test Strips ³
Apricot	475 ± 30	300 ± 75	246
Coconut	6 ± 0.4	6 ± 16	ND ⁴
Ginger	117 ± 3	142 ± 79	116
Mango	202 ± 5	206 ± 131	ND
Papaya	35 ± 1	18 ± 10	ND
Pineapple	256 ± 8	170 ± 12	113
Potato	30 ± 2	16 ± 14^5	83
Tomato	2429 ± 169	2054 ± 435	6358
White Grape Juice	20 ± 1	7 ± 7	ND
White Wine Vinegar	15 ± 1	16 ± 6	ND

^{1,2} Samples were analyzed in triplicate from the same homogenate. Average sulfite concentration in mg/kg SO_2 and standard deviation are reported.

³ Samples were analyzed in triplicate from the same homogenate. Since the method is semi-quantitative, the most commonly occurring response was reported after adjustments for dilution. Concentrations reported as mg/kg SO_2 .

⁴ ND: not able to be detected.

⁵ Sample analyzed using manufacturer's recommended extraction for starch samples. Sample was also analyzed using the dried fruit protocol and the concentration was determined to be 12 ± 3 mg/kg SO_2 .

sulfite concentrations showing that reproducibility can be a problem especially at concentrations less than 20 ppm SO_2 . Sample preparation steps also seemed to affect the reproducibility of the method. Those matrices that were not diluted and added in pure form to the cuvette had higher relative standard deviations compared to those samples which were diluted. Since this method is based on spectrophotometry it could be that without dilution, matrix components are able to interfere with the analysis and decrease the reproducibility of the method. Only 5 samples could be quantitatively analyzed with the test strips, either due to interference by matrix components or because no sulfite was detected in the samples. The colorimetric scale used for determining sulfite concentration ranges from a light pale pink to a dark orange red color. Any color present in the sample made determination of the true concentration difficult. This was true for the papaya and the mango which both contained yellow dyes in their ingredient list. The other three samples where sulfites were not detected were the white grape juice, coconut, and white wine vinegar which had concentrations between 6 and 20 ppm SO_2 as determined by LC-MS/MS. These results could indicate that the test strips are not able to be used for low sulfite concentrations including at the regulatory labeling threshold of 10 ppm SO_2 .

The LC-MS/MS method has been thoroughly validated in previously

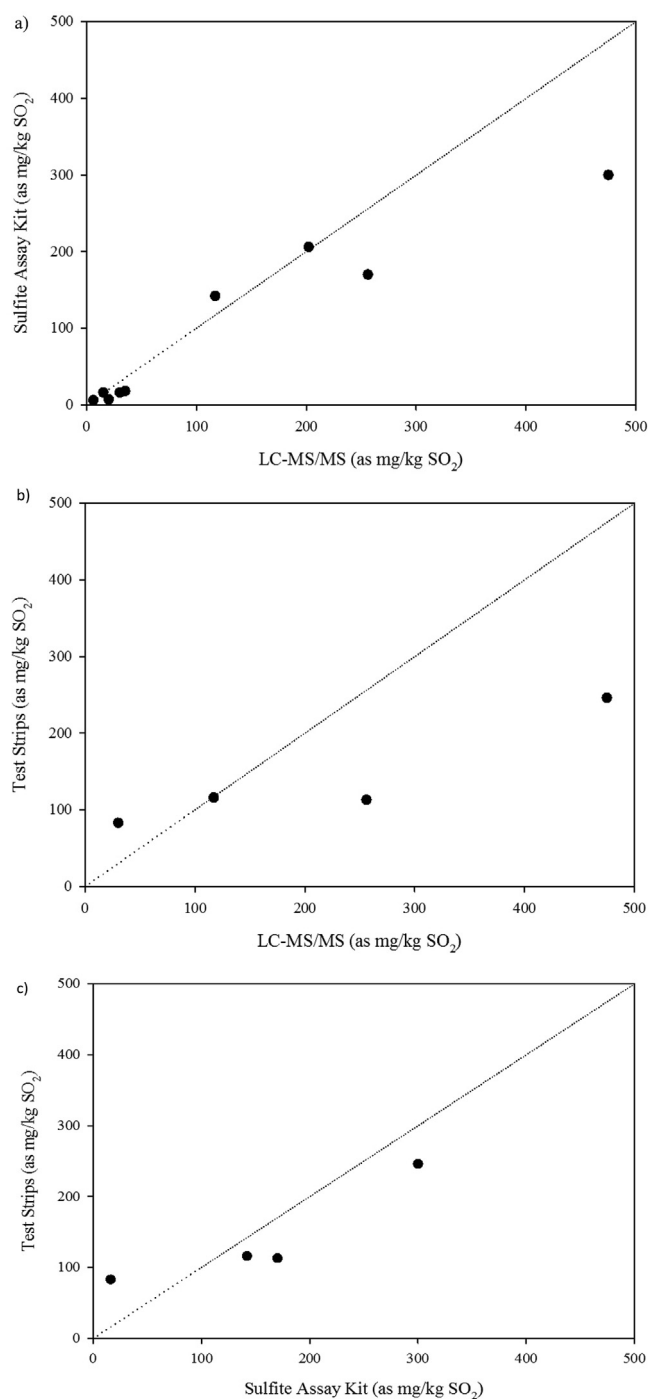


Fig. 2. Correlation plots comparing the responses of the three test methods. The line displayed on the plot shows $y = x$ or where the correlation coefficient would be 1. The tomato data points were removed from the plots due to their difference in magnitude compared to the other matrices.

published studies (Carlos & de Jager, 2017; Robbins et al., 2015). The results of the 11-laboratory validation include average recoveries ranging from 86 to 114% with % RSD_r and % RSD_R of 4.5–17.5% and 8.6–22.5%, respectively. For this reason, the authors did not do spike recovery studies with the rapid assays but instead compared the results of those assays with the LC–MS/MS method and the other assays. To better compare the three methods, correlation plots were created (Fig. 2). The results of the sulfite assay kit and the LC–MS/MS method compared well to each other especially at low concentrations. Although the results are similar it should be noted that the relative standard

deviation was quite large especially with those samples under 20 ppm SO₂ for the sulfite assay kit. Further investigation with other sulfited matrices is necessary especially with those that contain more than 500 ppm SO₂ because only one matrix in this study was in this category. The LC–MS/MS and sulfite assay kit correlated best with each other as opposed to when they were compared with the test strips. The test strips appear to be biased low compared to the other two methods. In other words, the concentration determined by test strip was significantly lower than the determined concentrations from both the LC–MS/MS and sulfite assay kit methods.

4. Conclusions

Further investigation into more matrices and additional samples of those matrices reported here is necessary for a full evaluation. Prior to utilizing these methods, it is recommended that a full validation be completed to establish proper LOQs and LODs in matrices of interest. This initial investigation shows that these rapid analysis kits do not offer a method that is as accurate nor as repeatable over as broad a range of concentrations as the LC–MS/MS method. The sulfite assay kit shows the most promise for use as a quick test but the method displayed large %RSD values for some matrices investigated and still requires the analyst to have access to a spectrophotometer which rules out this method for use by consumers. However, this method could potentially be used by food manufacturers interested in a rapid test for sulfite determination. Prior to beginning use, further investigation into specific matrices should be completed to ensure that the test is applicable to the specific matrix and sulfiting concentration investigated. The test strips appear to be more accurate at concentrations greater than 50 ppm SO₂ so they could be used for consumers or food manufacturers to test food with high concentrations of sulfite. However, they would not be able to warn consumers or food processors of the presence of sulfite at concentrations close to the regulatory labeling threshold.

Declaration of interests

The authors declare that they have no known competing financial interests

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