Application Note

Pharmaceuticals

Measuring Total Sulfite (SO_2) in Sucrose According to the USP43-NF38-6076 Method

Cary 3500 UV-Vis spectrophotometer offers time savings and reduced error sources.

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Introduction

Pharmaceutical excipients are included in pharmaceutical formulations not for their therapeutic activity but to enhance the manufacturing process, aid in stability or bio-availability and improve patient acceptability (*1*). Sucrose ($C_{12}H_{22}O_{11}$) is the most commonly used sweetener in the pharmaceutical industry. Sucrose is used to mask the unpleasant taste of oral medicines, act as a preservative, and enhance the viscosity of liquid medicines.

To ensure safety and quality of sucrose used in pharmaceutical formulations, accurate determination of impurities is required. Sulfites are a common impurity in sucrose. The United States Pharmacopeial method USP43-NF38-6076 (*2*) describes an enzymatic method to determine total sulfite content in sucrose using UV-Vis spectrophotometry.

In this study, we demonstrate the benefits of the Agilent Cary 3500 UV-Vis spectrophotometer in determining total sulfite in sucrose according to the USP method.

Experimental

Background

The USP43-NF38-6076 method uses the fact that sulfite is oxidized by sulfite oxidase to create sulfate and hydrogen peroxide in the presence of oxygen, as shown in the reaction equation following.

$$SO_3^{2-} + O_2 + H_2O \xrightarrow{\text{(Sulfite oxidase)}} SO_4^{2-} + H_2O_2 \quad (1)$$

The hydrogen peroxide formed is then reduced by nicotinamide–adenine-dinucleotide–peroxidase (NADH peroxidase) in the presence of reduced nicotinamide–adenine dinucleotide (NADH), as in equation 2, following.

$$H_2O_2 + NADH + H^+ \xrightarrow{\text{(NADH peroxidase)}} 2H_2O + NAD^+ \quad (2)$$

The amount of NAD^+ formed in the reaction is directly proportional to the amount of sulfite in the sample. The NADH consumption can be determined by measuring the absorbance at 340 nm.

Total sulfite concentration can be determined by calculating the difference (A_1-A_2) for both blank and samples. A_1 is the absorbance at the start of the enzymatic reaction. A_2 is the absorbance at the end of the reaction. To obtain $\Delta A_{sulfite}$ the absorbance difference ($A_1 - A_2$) of the blank is subtracted from the absorbance difference ($A_1 - A_2$) of samples.

Sulfite concentration as SO_2 [g/L] can be calculated as follows:

$$C\ (total\ SO2) = \frac{V \times Mol.Wt}{\varepsilon \times d \times v} \times\ \Delta\text{A Sulfite} \quad (3)$$

Where:

V = final volume [mL]

Mol.Wt = molecular weight of SO_2 [g/mol]

ε = extinction coefficient of NADH at 340 nm = 6300 [l x mol^{-1} x cm^{-1}]

d = pathlength [cm]

v = sample volume [mL]

Experimental

Sample preparation

Sample 1 and 2 solutions: To prepare sample 1, 2.0 grams of sucrose (Sigma Aldrich, CAS Number 57-50-1) was dissolved in 5.0 mL of distilled water to yield a final concentration of 400 mg/mL. This procedure was repeated for sample 2, this time using 2.0 grams of commercial sucrose (obtained from local supermarket).

Sulfite standard solution (80 ppm SO_2): 157.5 mg of anhydrous sodium sulfite was dissolved in 1.0 L distilled water to yield a final concentration of 0.1575 mg/mL.

Reference solution: 4.0 grams of sucrose (Sigma Aldrich, CAS Number 57-50-1) was dissolved in 5.0 mL distilled water. 0.5 mL of the sulfite standard solution described above was then added, with the resultant solution then diluted with distilled water to 10.0 mL.

Standard solutions: 1.0 g of citric acid was added to a 1.0 L volumetric flask and dissolved with 1.0 L distilled water. After that, 800 mg of anhydrous sodium sulfite (Merck, CAS Number 7757-83-7) was accurately weighted and added to the solution (final conc ~400 mg/L as SO_2). Multiple sulfite standards (0 – 400 mg/L, Table 1) were prepared by diluting the stock solution with the appropriate volume of 1 g/L citric acid solution.

Table 1. Standard solutions concentration

Standard No.	Concentration (mg/L)
Standard 1	0
Standard 2	67
Standard 3	135
Standard 4	203
Standard 5	270
Standard 6	338
Standard 7	407

Enzymatic kit: Total sulfite (SO_2) enzymatic kit, Table 2 (Megazyme, Product code: K-ETSULPH).

Table 2. Megazyme enzymatic kit contents.

Bottle No.	Ingredient
1	Buffer
2	NADH
3	NADH peroxidase suspension
4	Sulfite oxidase suspension

Cuvettes: Standard, 3.5 mL 10 mm optical pathlength quartz cuvettes were used for this experiment with star type magnetic stirrers.

Instrumentation and method

An Agilent Cary 3500 Multicell UV-Vis spectrophotometer was used for all measurements (Figure 1). The method parameters are shown in Table 3.

Figure 1. Cary 3500 Multicell UV-Vis spectrophotometer.

Table 3. Instrument parameters. To monitor NADH consumption, the absorbance at a single wavelength was measured over time (parameters 1). To measure NADH consumption before and after the reaction, the absorbance of the solution was repeatedly measured over a wavelength range (parameters 2). The instructions supplied with the enzymatic kit recommended using air to determine a baseline measurement.

Parameter	Parameters 1	Parameters 2
Wavelength (nm)	340	300 to 400
Signal Averaging Time (s)	5	0.2
Data Interval	Not applicable	1
Spectral Bandwidth (nm)	2	2
Stirring Speed (rpm)	500	500
Baseline	Air	Air
Time (min)	45	4
Speed rate (nm/min)	Not applicable	300
Time interval between scans (s)	Not applicable	0

Each cuvette was filled with a total volume of 3.340 mL, as described in Table 4, using a pipette. A star stirrer was placed inside each cuvette before capping. Samples were mixed using the stirring feature of the Cary 3500 at 500 rpm. Absorbances of the solutions (A1) were measured after approx. 4 min of stirring. The reaction was then started with the addition of sulfite oxidase. Absorbances of the solutions (A2) were measured at the end of the reaction after 45 min. The Cary 3500 is suited to this work, with its software-controlled in-cuvette stirring functionality ensuring sample homogeneity. This functionality replaces mixing with a spatula or mixing by inverting the cuvette, which might cause sample loss and contamination.

Table 4. The volume of each reagent solution and distilled water added to each cuvette to prepare samples, references, and standards.

Solution	Blank	Sample	Reference	Standards (0 to 407 ppm)
Distilled water	2.60 mL	0.6 mL	0.6 mL	2.50 mL
Solution to be measured	-	2.0 mL	2.0 mL	0.10 mL
Buffer	0.50 mL	0.50 mL	0.50 mL	0.50 mL
NADH	0.20 mL	0.20 mL	0.20 mL	0.20 mL
NADH peroxidase	0.02 mL	0.02 mL	0.02 mL	0.02 mL
Sulfite oxidase	0.02 mL	0.02 mL	0.02 mL	0.02 mL

The concentration of each of the seven standards is shown in Table 1. The measurement was conducted using the optional Agilent OpenLab software with the Cary UV Workstation software that is provided with the instrument. OpenLab offers tools that help comply with 21 CFR Part 11 and Annex 11 data integrity requirements. Those tools include a searchable audit trail of every change made.

Results and discussion

Quantitative analysis of total sulfites in sucrose samples according to the USP method

To determine total sulfite content (SO_2) in sucrose according to the USP43-NF38 monograph, the absorbance at the maximum that occurs at about 340 nm should be recorded at the start (A_1) and end (A_2) of the reaction time. These values are then subtracted from the corresponding values obtained with the blank solution. The absorbance difference ($A_1 - A_2$) for the sample solution should be not more than half the absorbance difference of the reference solution's absorbance values.

The $\Delta A_{sulfite}$ in the sucrose samples measured using the Agilent Cary 3500 UV-Vis spectrophotometer was 0.0023 Abs and 0.0044 Abs for samples 1 and 2 respectively (Table 5). The $\Delta A_{sulfite}$ for both samples was less than half of the reference and within the acceptance range specified in the USP. The results suggest that the Cary 3500 is suitable for the determination of sulfite impurities in sucrose.

Table 5. Determination of total sulfite (SO_2) in sucrose samples.

Entry	A_1	A_2	A_1-A_2	$\Delta A_{sulfite}$	Total Sulfite (g/L)	Total Sulfite (ppm)
Sample 1	1.8805	1.6444	0.2360	0.0069	0.0023	2.3671
Sample 2	1.8951	1.6529	0.2422	0.0131	0.0044	4.4722
Reference	1.8957	1.4572	0.4385	0.2094	0.0711	71.125

Quantitative analysis of total sulfites in standard solutions

The Cary 3500 Multicell UV-Vis instrument allows simultaneous measurement of eight cuvette positions (seven samples and one reference). Seven standards were monitored at the same time under the same conditions by performing a wavelength scan from 300 to 400 nm, 12 times: before, during and after the reaction started (Figure 2). The ability to measure multiple samples/standards at the same time eliminates environmental and operator-generated analytical variables and the resultant risk to data accuracy. The Cary UV Workstation kinetic application allows the analyst to choose either a discrete number of wavelengths or a wavelength range to scan.

The Absorbance (A_1) of each of the seven standards was measured at the same time, using the Cary UV Workstation Kinetics application. The measurement was performed by measuring the absorbance between 300 to 400 nm for 4 minutes (12 scans). After that, 20 µL of sulfite oxidase suspension was added to each standard sequentially using a pipette and mixed. The NADH consumption was monitored at 340 nm by the decrease in absorbance for 45 minutes using the Kinetics application. The absorbance of the standard solutions (A_2) was measured at the end of the reaction using the same parameters described in Table 3 (parameters 2) measuring Absorbance (A_1). Total Sulfite (SO_2) was calculated as described in equation 3, using data exported to MS Excel. The results are summarized in Table 6.

Table 6. Calculated total sulfite (SO_2) concentrations for each standard solution.

Standard solution	A_1	A_2	A_1-A_2	$\Delta A_{sulfite}$	Total Sulfite [g/L]	Total Sulfite [ppm]	Total Sulfite [ppm] as prepared
1	1.7916	1.5963	0.1952	0.0001	0.0001	0.0000	0.0000
2	1.8303	1.4371	0.3932	0.1979	0.0672	67.2190	67.5254
3	1.8364	1.2289	0.6075	0.4123	0.1400	140.0200	135.4576
4	1.8419	1.0260	0.8159	0.6206	0.2107	210.7690	203.3898
5	1.8011	0.7785	1.0226	0.8273	0.2809	280.9740	270.9152
6	1.8554	0.6322	1.2232	1.0279	0.3490	349.0930	338.8474
7	1.9025	0.5076	1.3949	1.1996	0.4073	407.3990	406.7796

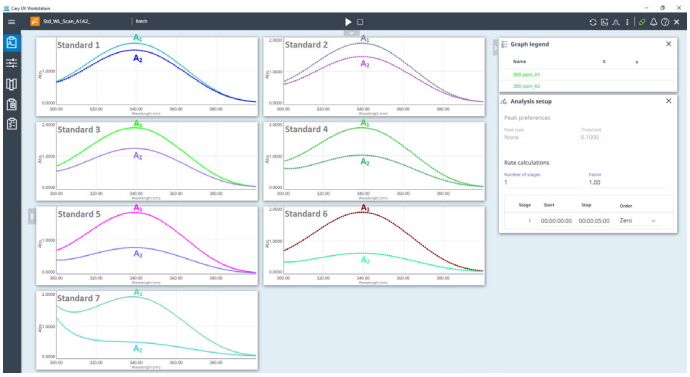

Figure 2. The Cary UV Workstation Kinetics application, combined with the Cary 3500 Multicell UV-Vis instrument, allows the measurement of seven standards simultaneously. Shown here are the overlaid wavelength scans (300 to 400 nm) of each of the seven standards at the start of the reaction (labeled A_1) and after the reaction is complete (labeled A_2).

Figure 3. Time-based absorbance measurements of seven standard solutions, measured at the same time using the Cary 3500 UV-Vis system.

Monitoring the consumption of NADH over time

The Cary UV Workstation Kinetics application was used to monitor the consumption of NADH after the addition of sulfite oxidase. All seven standards were measured at the same time, with the instrument monitoring the absorbance at 340 nm of each standard for 45 minutes (Figure 3). This approach offers considerable time savings, compared to measuring each standard separately, measurements which would have taken over 5 hours to complete. The Cary 3500 has a high-speed (up to 250 data points/second) data collection rate and a wide photometric range with no moving parts. These features ensure that accurate data is collected from all measurement types.

Conclusion

The Cary 3500 Multicell UV-Vis spectrophotometer was able to monitor the NADH consumption in seven standards monitored simultaneously, in a single experiment. This approach saved over five hours of measurement time, compared to measuring each standard individually. The simultaneous measurement also meant that all seven standards were measured under the same conditions, removing any measurement variables such as changes in ambient temperature.

The total sulfite content in sucrose was determined, according to the USP43-NF38 method. The measurement of multiple samples simultaneously reduces possible environmental, operator, and instrument sources of error and improves data quality. The optional Agilent OpenLab software was used to support Part 11/Annex 11 compliance for the data collection process.

The software-controlled stirring capability of the Cary 3500 mixed all reactants in the cuvette throughout the measurement time.

References

1. Lucie Nováková et al., Pharmaceutical Analysis | Overview, Encyclopedia of Analytical Science (Third Edition), Academic Press, 2019, Pages 200-218, https://doi.org/10.1016/B978-0-12-409547-2.14504-4.
2. United States Pharmacopeia, USP43-NF38-6076.

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