Megazyme

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TOTAL SULPHITE (SO_2)

(*Enzymatic*)

ASSAY PROCEDURE

K-ETSULPH 06/21

(*50 Manual Assays per Kit) or
(588 Auto-Analyser Assays per Kit) or
(500 Microplate Assays per Kit)

** The number of tests per kit can be doubled if all volumes are halved*

INTRODUCTION:

Sulphur dioxide (SO_2) is used widely as an additive in various forms, most commonly as sulphites (or sulfites), in the wine, beverage and food industries where it acts primarily as an antimicrobial and antioxidant preservative.

During wine making, sulphites are used as an essential additive, usually at post-malolactic fermentation, in the control of contamination by *Brettanomyces* during ageing and also to protect the wine against detrimental "oxidative and enzymatic browning". SO_2 is only active as an antimicrobial and antioxidant preservative in the unbound "free" form. Given that SO_2 becomes "inactive" when it binds the colour pigments of wine, and with legal restrictions on SO_2 levels in wine, it has become valuable to wine producers to measure both the Free SO_2 and Total SO_2. In addition to this, due to the increased awareness of the adverse effects of sulphites and the prevalence of sulphite intolerance in some individuals, sulphite levels in foods and drinks are strictly regulated by various governing bodies and therefore, there is a requirement for accurate determination of the level of sulphites in foods, beverages and wines.

This kit (**K-ETSULPH**) is suitable for the specific measurement of "Total Sulphite" as SO_2 in wines, beverages, foodstuffs and other materials.

PRINCIPLE:

Sulphite is oxidised to sulfate by the enzyme, sulfite oxidase, in the presence of oxygen (1).

$$(1)\ SO_3^{2-} + O_2 + H_2O \xrightleftharpoons{\text{(sulphite oxidase)}} SO_4^{2-} + H_2O_2$$

Hydrogen peroxide formed in this reaction is reduced in the presence of the enzyme, NADH peroxidase and NADH (2).

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

The amount of NAD^+ formed in the above reaction pathway is stoichiometric with the amount of sulphite or aldehyde-bound sulphite. It is NADH consumption which is measured by the decrease in absorbance at 340 nm.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

Sulfite oxidase will react with sulphite, isothiocyanates and isothiocyanate glycosides while sulfates, thiosulfates, sulfinic acid compounds and sulfides do not react.

Sulfonic acid compounds can cause a creep reaction under these assay conditions.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to a sulphite concentration of 3.4 mg/L of sample solution with a sample volume of 0.1 mL. The

detection limit is 0.34 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 2.0 mL.

The assay is linear over the range of 1 to 50 µg of sulphite per assay. With a sample volume of 0.1 mL, this corresponds to a sulphite concentration of 10 to 500 mg/L of sample solution. In duplicate determinations, using one sample solution, an absorbance difference of 0.005 to 0.010 may occur. With a sample volume of 0.1 mL, this corresponds to a sulphite concentration of 1.7 to 3.4 mg/L of sample solution. If the sample is diluted during sample preparation, the result is multiplied by the dilution factor, F. If, in sample preparation, the sample is weighed, e.g. 10 g/L, a difference of 0.02 to 0.05 g/100 g can be expected.

INTERFERENCE:

The presence of L-ascorbic acid will slow the sulfite oxidase reaction and L-ascorbic acid levels higher than 50 µg per manual assay should be removed during sample preparation (see Section (d), page 10).

Due to the characteristic instability of aqueous sulphite samples, recoveries of less than 100% should be expected. This is consistent with values obtained with other commercially available enzymatic sulphite test kits.

SAFETY:

The general safety measures that apply to all chemical substances should be adhered to.
For more information regarding the safe usage and handling of this product please refer to the associated SDS that is available from the Megazyme website.

KITS:

Kits suitable for performing 50 assays in manual format (or 588 assays in auto-analyser format or 500 assays in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1:	Buffer (28 mL; pH 8.0) plus sodium azide (0.02% w/v) as a preservative. Stable for > 2 years at 4°C.
Bottle 2:	NADH. Freeze dried powder. Stable for > 2 years below -10°C.
Bottle 3:	NADH peroxidase solution (1.1 mL). Stable for > 2 years below -10°C.
Bottle 4:	Sulfite oxidase suspension (1.1 mL). Stable for > 2 years at 4°C.
Bottle 5:	Sodium sulphite standard powder (~ 5 g). Stable for > 5 years; store sealed at room temperature.

PREPARATION OF REAGENT SOLUTIONS (SUPPLIED):

1. Use the contents of bottle 1 as supplied.
Stable for > 2 years at 4°C.

2. Dissolve the contents of bottle 2 in 11 mL of distilled water. **Stable for ~ 4 weeks at 4°C** or stable for > 2 years below -10°C (to avoid repetitive freeze/thaw cycles, divide into appropriately sized aliquots and store in polypropylene tubes).

3 & 4. Use the contents of bottles 3 and 4 as supplied. Before opening for the first time, shake the bottle to remove any enzyme that may have settled on the rubber stopper. Subsequently, store the bottle in an upright position. **Swirl the bottle to mix contents before use.**
Bottle 3: Stable for > 2 years below -10°C.
Bottle 4: Stable for > 2 years at 4°C.

5. Weigh 1 g of citric acid into a 1 L volumetric flask, make to 1 L with distilled water and dissolve. Accurately add 590 mg of sodium sulphite, dissolve and use directly in the assay. This sulphite solution is equivalent to 300 mg/L as SO_2. Sulphite in solution is not stable and will lose ~ 2% of the sulphite content per h, therefore **this solution must be made fresh on the day of use**.

NOTES:

1. The sulphite standard solution is only assayed where there is some doubt about the accuracy of the spectrophotometer being used or where it is suspected that inhibition is being caused by substances in the sample. The concentration of sulphite is determined directly from the extinction coefficient of NADH (see page 5).
2. Only use fresh distilled water for the assay. Alternatively, treat 100 mL of demineralised water with 1 g of activated charcoal for 5 min with stirring and then filter.

EQUIPMENT (RECOMMENDED):

1. Volumetric flask (1 L).
2. Disposable plastic or glass cuvettes (1 cm light path, 3 mL).
3. Micro-pipettors, e.g. Gilson Pipetman® (20 µL, 200 µL and 1 mL).
4. Positive displacement pipettor, e.g. Eppendorf Multipette® - with 25 mL Combitip® (to dispense 0.5 mL aliquots of buffer 1 and 0.2 mL aliquots of NADH solution).
5. Stop clock.
6. Spectrophotometer set at 340 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Thermostated water bath.

A. MANUAL ASSAY PROCEDURE:

Wavelength: 340 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 3.34 mL
Sample solution: 1.0-50 µg of sulphite per cuvette (in 0.10-2.00 mL sample volume)

Read against air (without a cuvette in the light path) or against water

Pipette into cuvettes	Blank	Sample
distilled water (at ~ 25°C)	2.60 mL	2.50 mL
sample solution	-	0.10 mL
solution 1 (buffer)	0.50 mL	0.50 mL
solution 2 (NADH)	0.20 mL	0.20 mL
solution 3 (NADH peroxidase)	0.02 mL	0.02 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:		
suspension 4 (sulfite oxidase)	0.02 mL	0.02 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 30 min). If the reaction has not stopped after 30 min, continue to read the absorbances at 10 min intervals until the absorbances either plateau or decrease constantly over 10 min**.		

* for example with a plastic spatula or by gentle inversion after sealing the cuvette with a cuvette cap or Parafilm®.

** if this "creep" rate is greater for the sample than that of the blank, extrapolate the absorbances (sample and blank) back to the time of the addition of suspension 4 (sulfite oxidase).

NOTE: Due to the characteristic instability of sulphite solutions, samples should be analysed as soon as possible after sample preparation.

CALCULATION (Manual Assay Procedure):

Determine the absorbance difference (A_1-A_2) for both blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample, thereby obtaining $\Delta A_{sulphite}$.

The value of $\Delta A_{sulphite}$ should be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of sulphite as SO_2 can be calculated as follows:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A_{sulphite} \quad [g/L]$$

where:

V = final volume [mL]
MW = molecular weight of SO_2 [g/mol]
ε = extinction coefficient of NADH at 340 nm
= 6300 [l x mol^{-1} x cm^{-1}]
d = light path [cm]
v = sample volume [mL]

It follows for sulphite as SO_2:

$$c = \frac{3.34 \times 64.06}{6300 \times 1 \times 0.1} \times \Delta A_{sulphite} \quad [g/L]$$

$$= 0.3396 \times \Delta A_{sulphite} \quad [g/L]$$

If the sample has been diluted during preparation, the result must be multiplied by the dilution factor, F.

When analysing solid and semi-solid samples which are weighed out for sample preparation, the content (g/100 g) is calculated from the amount weighed as follows:

Content of sulphite as SO_2:

$$= \frac{c_{sulphite}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

NOTE: These calculations can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

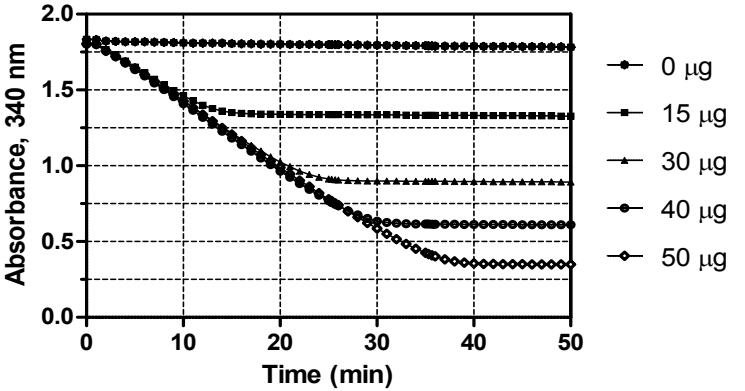

Figure 1. Decrease in absorbance at 340 nm on incubation of 0-50 µg of sulphite (as SO_2) with sulfite oxidase and NADH peroxidase in the presence of NADH at 25°C using 1 cm path-length cuvettes (Manual Format; page 4).

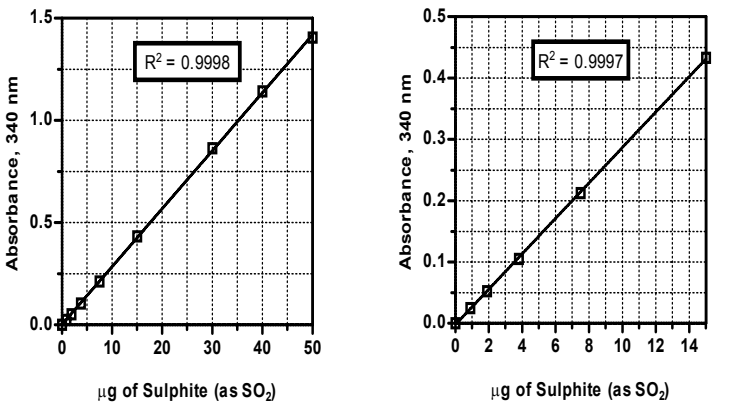

Figure 2. Calibration curves showing the linearity of **K-ETSULPH** from 0-50 µg of sulphite (as SO_2). The reactions used to generate this calibration curve were performed at 25°C using 1 cm path-length cuvettes (Manual Format; page 4).

B. AUTO-ANALYSER ASSAY PROCEDURE:

NOTES:

1. The Auto-Analyser Assay Procedure for sulphite can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of sulphite **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Preparation of R1:

This kit is suitable for the preparation of 162 mL of reagent (equivalent to 588 reactions of 0.275 mL). Reagent preparation is performed as follows:

Component	Volume
distilled water (at ~ 25°C) solution 1 (buffer) solution 2 (NADH) solution 3 (NADH peroxidase)	24.3 mL 5.50 mL 2.20 mL (after adding 11 mL of H_2O to bottle 2) 0.22 mL
Total volume	32.22 mL

Preparation of R2:

Component	Volume
distilled water (at ~ 25°C) suspension 4 (sulfite oxidase)	3.00 mL 0.22 mL
Total volume	3.22 mL

EXAMPLE METHOD:

R1:	0.250 mL
Sample:	~ 0.01 mL
R2:	0.025 mL
Reaction time:	~ 30 min at 25°C
Wavelength:	340 nm
Prepared reagent stability:	> 7 days when refrigerated
Calculation:	endpoint
Reaction direction:	decrease
Linearity:	10-500 mg/L of sulphite as SO_2 using 0.01 mL sample volume

C. MICROPLATE ASSAY PROCEDURE:

NOTES:

1. The Microplate Assay Procedure for sulphite can be performed using either a single point standard or a full calibration curve.
2. For each batch of samples that is applied to the determination of sulphite **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**

Wavelength: 340 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C
Final volume: 0.334 mL
Linearity: 0.1-5 µg of sulphite per well (in 0.01-0.20 mL sample volume)

Pipette into wells	Blank	Sample	Standard
distilled water (at ~ 25°C)	0.260 mL	0.250 mL	0.250 mL
sample solution	-	0.010 mL	-
standard solution	-	-	0.010 mL
solution 1 (buffer)	0.050 mL	0.050 mL	0.050 mL
solution 2 (NADH)	0.020 mL	0.020 mL	0.020 mL
solution 3 (NADH peroxidase)	0.002 mL	0.002 mL	0.002 mL
Mix*, read the absorbances of the solutions (A_1) after approx. 4 min and start the reactions by addition of:			
suspension 4 (sulfite oxidase)	0.002 mL	0.002 mL	0.002 mL
Mix* and read the absorbances of the solutions (A_2) at the end of the reaction (approx. 30 min). If the reaction has not stopped after 30 min, continue to read the absorbances at 10 min intervals until the absorbances either plateau or decrease constantly over 10 min.			

* for example using microplate shaker, shake function on a microplate reader or repeated aspiration (e.g. using a pipettor set at 50-100 µL volume).

CALCULATION (Microplate Assay Procedure):

$$g/L = \frac{\Delta A_{sample}}{\Delta A_{standard}} \times g/L \text{ standard} \times F$$

If the sample is diluted during preparation, the result must be multiplied by the dilution factor, F.

SAMPLE PREPARATION:

NOTE: Due to the characteristic instability of sulphite solutions, samples should be analysed as soon as possible after sample preparation.

1. Sample dilution.

The amount of sulphite present in the cuvette (i.e. in the 0.1 mL of sample being analysed) should range between 1 and 50 µg. The sample solution must therefore be diluted sufficiently to yield a concentration of sulphite between 10 and 500 mg/L.

Dilution table

Estimated concentration of sulphite (mg/L)	Dilution with water	Dilution factor (F)
< 500	No dilution required	1
500-5000	1 + 9	10
> 5000	1 + 99	100

If the value of $\Delta A_{sulphite}$ is too low (e.g. < 0.100), weigh out more sample or dilute less strongly. Alternatively, the sample volume to be pipetted into the cuvette can be increased to 2.0 mL, making sure that the sum of the sample and distilled water components in the reaction is 2.60 mL and using the new sample volume in the equation.

2. General considerations.

(a) Liquid samples: clear, slightly coloured and approximately neutral, liquid samples can be used directly in the assay.

(b) Acidic samples: if > 0.1 mL of an acidic sample is to be used undiluted, the pH of the solution should be increased to approx. 8.0 using 2 M NaOH and the solution incubated at room temperature for 30 min.

(c) Carbon dioxide: samples containing carbon dioxide should be degassed by increasing the pH to approx. 8.0 with 2 M NaOH and gentle stirring, or by stirring with a glass rod.

(d) Strongly coloured samples: if used undiluted, strongly coloured samples should be treated by the addition of 0.2 g of polyvinylpolypyrollidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper or centrifuge at 4,000 x *g* for 10 min.

(e) Solid samples: homogenise or crush solid samples in distilled water and filter if necessary.

(f) Samples containing fat: extract such samples with hot water

at a temperature above the melting point of the fat, e.g. in a 100 mL volumetric flask at 60°C. Adjust to room temperature and fill the volumetric flask to the mark with distilled water. Store on ice or in a refrigerator for 15-30 min and then filter. Discard the first few mL of filtrate and use the clear supernatant (which may be slightly opalescent) for the assay.

(g) Samples containing protein: deproteinise samples containing protein by adding an equal volume of ice-cold 1 M perchloric acid with mixing. Filter or centrifuge at 1,500 *g* for 10 min and adjust the pH of the supernatant to approx. 8.0 with 1 M KOH. Use the supernatant in the assay after appropriate dilution.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of sulphite in white wine.

The sulphite concentration of white wine can be determined without any sample treatment except dilution (if required). *Typically use a sample volume of 0.1 mL.*

(b) Determination of sulphite in red wine.

Adjust the pH of approx 25 mL of red wine to pH 8.0 and dilute 2-fold with distilled water. *Typically use a sample volume of 0.1 mL.* Alternatively, adjust the pH of approx. 25 mL of red wine to pH 8.0 then add of 0.5 g of polyvinylpolypyrolidone (PVPP). Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper or centrifuge at 4,000 x *g* for 10 min. Dilute the filtrate/ supernatant 2-fold with distilled water and use directly in the assay. *Typically use a sample volume of 0.1 mL.*

(c) Determination of sulphite in beer.

Filter the beer sample immediately through Whatman No. 1 filter paper then add 0.7 g of bentonite to 10 mL beer in a 50 mL beaker with stirring for 1 min. Filter through Whatman No. 1 filter paper and use the filtrate in the assay. *Typically use a sample volume of 0.5-1.0 mL.*

(d) Determination of sulphite in fruit juice.

Clarify turbid juice by filtration through Whatman No. 1 filter paper or centrifugation at 4,000 x *g* for 10 min.

L-Ascorbic acid must be removed from samples if present at concentrations > 100 mg/L. Adjust the pH of 2 mL of clarified juice to approx 6.0 with 2 M NaOH (the volume of NaOH used must be accounted for in the final dilution factor of the sample). Add approx. 20 U of ascorbate acid oxidase and incubate for 10 min. Adjust the pH of the sample to pH 8.0 with 2 M NaOH.

For coloured juices, add of 0.1 g of polyvinylpolypyrollidone (PVPP), shake the tube vigorously for 2 min and filter through Whatman

No. 1 filter paper or centrifuge at 4,000 x *g* for 10 min. Use the clear, slightly coloured filtrate/supernatant in the assay. *Typically use a sample volume of 0.1 mL.*

(e) Determination of sulphite in potatoes.

Accurately weigh approx. 5 g of dried potato and homogenise using a mortar or blender. Transfer the sample to a 100 mL volumetric flask and add approx. 60 mL of hot (~ 70°C) distilled water with vigorous shaking or stirring for 5 min. Store on ice or in a refrigerator for 15-30 min. Fill up to mark with distilled water, mix thoroughly and filter through Whatman No. 1 filter paper or centrifuge. at approx. 4,000 x *g* for 10 min. Use the filtrate/supernatant in the assay after appropriate dilution (if required).

REFERENCE:

Beutler, H. O. (1988). Sulphite. *"Methods of Enzymatic Analysis"* (Bergmeyer, H. U., ed.), 3rd ed., **Vol. VII**, pp. 585-591, VCH Publishers (UK) Ltd., Cambridge, UK.

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