TOTAL AND FREE SULFITE (LIQUID READY)

ASSAY PROTOCOL

K-SULPH

05/22

(40 Manual Assays per Kit) or
(400 Auto-Analyser Assays per Kit) or
(400 Microplate Assays per Kit)

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CONTENTS:

INTRODUCTION:

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

During wine making sulfites are used as an essential additive, usually at post-malolactic fermentation, in the control of contamination by *Brettanomyces* during aging 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 (FSO_2) and Total SO_2 (TSO_2). In addition to this, due to the increased awareness of the adverse effects of sulfites and the prevalence of sulfite intolerance in some individuals, sulfite 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 sulfites in foods, beverages and wines.

This kit (**K-SULPH**) is suitable for the specific measurement of both "Total Sulfite" and "Free Sulfite" especially in wines, beverages, foodstuffs and other materials.

PRINCIPLE:

The Total Sulfite assay is based on the reaction principle between thiol groups and Ellman's reagent.

The Free Sulfite assay is based on the reaction principle of SO_2, fuchsin and aldehyde binding.

SPECIFICITY, SENSITIVITY, LINEARITY AND PRECISION:

Total Sulfite (TSO_2)

Compounds containing free thiols (e.g. cysteine, β-mercaptoethanol and dithiothreitol) can react in the TSO_2 assay.

The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to a TSO_2 concentration of ~ 2.64 mg/L of sample solution at a sample volume of 0.05 mL. The detection limit is ~ 5.28 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 0.05 mL.

The assay is linear over the range of 0.25 to 20 µg of TSO_2 per assay. With a sample volume of 0.05 mL, this corresponds to a TSO_2

concentration of ~ 5 to 400 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 0.05 mL, this corresponds to a TSO_2 concentration of ~ 1.32 to ~ 2.64 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.

Free Sulfite (FSO_2)
The smallest differentiating absorbance for the assay is 0.010 absorbance units. This corresponds to a FSO_2 concentration of ~ 1 mg/L of sample solution at a sample volume of 0.05 mL. The detection limit is ~ 2 mg/L, which is derived from an absorbance difference of 0.020 with a sample volume of 0.05 mL.

The assay is linear over the range of 0.25 to 7.5 µg of FSO_2 per assay. With a sample volume of 0.05 mL, this corresponds to a FSO_2 concentration of ~ 5 to 150 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 0.05 mL, this corresponds to a FSO_2 concentration of ~ 0.5 to ~ 1 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.

INTERFERENCES:

Total Sulfite (TSO_2)
Compounds containing free thiols (e.g. cysteine, β-mercaptoethanol and dithiothreitol) or thiol reactive compounds (e.g. aldehydes and maleimide compounds) can interfere with TSO_2 assay.

Aldehyde levels in wines will not cause interference of the TSO_2 assay.

Free Sulfite (FSO_2)
Thiol reactive compounds (e.g. aldehydes and maleimide compounds) can interfere with FSO_2 assay.

Endogenous aldehydes in wine are not considered as an interference for the FSO_2 assay for wine samples.

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 40 assays of each test in manual format (or 400 assays of each test in auto-analyser format or 400 of each test in microplate format) are available from Megazyme. The kits contain the full assay method plus:

Bottle 1: **Total Sulfite Reagent 1 (40 mL)**
Contains sodium azide (0.05% w/v) as a preservative. Store at room temperature. See individual label for expiry date.

Bottle 2: **Total Sulfite Reagent 2 (20 mL)**
Contains sodium azide (0.05% w/v) as a preservative. Store at room temperature. See individual label for expiry date.

Bottle 3: **Free Sulfite Reagent 1 (40 mL)**
Store at room temperature. See individual label for expiry date.

Bottle 4: **Free Sulfite Reagent 2 (20 mL)**
Store at room temperature. See individual label for expiry date.

Bottle 5: **Sulfite Standard**
Sodium Sulfite (5 g).
Store sealed at room temperature. See individual label for expiry date.

PREPARATION OF REAGENT SOLUTIONS (SUPPLIED):

1 - 4. Use the contents of bottles 1 to 4 as supplied.

5. Use the contents of bottle 5 as described for the preparation of TSO_2 and FSO_2 standards and calibration curves.

RECOMMENDED REAGENTS (NOT SUPPLIED):

1. Citric acid (Sigma cat. no. 251275).
2. Sodium Sulfite (Sigma cat. no. 71989).

EQUIPMENT (RECOMMENDED):

1. Disposable plastic cuvettes (1 cm light path, 3 mL).
2. Micro-pipettors, e.g. Gilson Pipetman® (200 µL and 1 mL).
3. Positive displacement pipettor, e.g. Eppendorf Multipette® - with 25 mL Combitip® [to dispense 0.5 mL and 1.0 mL aliquots of reagent solutions (solutions 1-4)].
4. Stop clock.
5. Analytical balance.
6. Spectrophotometer set at 405 nm and 575 nm.
7. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
8. Whatman No. 1 (9 cm) filter papers.

MANUAL ASSAY PROCEDURES:

NOTES:

1. The Manual Assay Procedure for TSO_2 or FSO_2 **must 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 TSO_2 or FSO_2 **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**
3. Prepare the TSO_2 or FSO_2 standards for the single point standard or full calibration curve as described for the Manual Assay Procedures for TSO_2 or FSO_2, respectively.
4. The calculation of TSO_2 or FSO_2 content, including the calibration curve analysis, can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

A. MANUAL ASSAY PROCEDURE FOR TOTAL SULFITE (TSO_2):

Wavelength: 405 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.55 mL
Sample solution: 0.25-20 µg of SO_2 per cuvette (5-400 mg/L) (in 0.05 mL sample volume)
Read against air (without cuvette in the light path) or against water

Pipette into cuvettes	Sample	Standard	Blank
distilled water (at ~ 25°C)	1.00 mL	1.00 mL	1.05 mL
Total Sulfite Reagent 1	1.00 mL	1.00 mL	1.00 mL
sample	0.05 mL	-	-
standard	-	0.05 mL	-
Mix* and read absorbances of the solutions (A_1) after exactly 3 min. Then add:			
Total Sulfite Reagent 2	0.50 mL	0.50 mL	0.50 mL
Mix* and read absorbances of the solutions (A_2) after exactly 3 min.			

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

PREPARATION OF THE TSO_2 SINGLE POINT STANDARD:

The TSO_2 standard is required for the calculation of TSO_2 in the test samples.

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 sulfite and dissolve. **This is the 300 mg/L standard, use this directly as the single point standard in the Manual Assay Procedure For Total Sulfite (TSO_2). Prepare on the day of use. Stable for 1 day at room temperature.**

NOTE: Alternatively, a full TSO_2 calibration curve can be used and the TSO_2 content calculated as described for "Preparation of the TSO_2 Calibration Curve".

CALCULATION OF TSO_2 CONTENT USING A SINGLE POINT STANDARD:

Determine the $\Delta A_{TotalSO_2}$ for the sample, standard and blank. Subtract the $\Delta A_{TotalSO_2}$ of the blank from that of the sample and the standard, thereby obtaining $\Delta A_{TotalSO_2\text{-}SAMPLE}$ and $\Delta A_{TotalSO_2\text{-}STD}$, respectively.

The value of $\Delta A_{TotalSO_2\text{-}SAMPLE}$ and $\Delta A_{TotalSO_2\text{-}STD}$ should be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of TSO_2 can be calculated as follows:

$$\Delta A_{TotalSO_2} = A_2 - A_1$$

$$c = \frac{\Delta A_{TotalSO_2\text{-}SAMPLE}}{\Delta A_{TotalSO_2\text{-}STD} / c_{TotalSO_2\text{-}STD}} \times F \quad [mg/L]$$

where:

$c_{TotalSO_2\text{-}STD}$ = concentration of TSO_2 standard (mg/L)
F = dilution factor

If the sample is 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 TSO_2

$$= \frac{c_{TotalSO_2}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

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).

PREPARATION OF THE TSO_2 CALIBRATION CURVE:

Weigh 1 g of citric acid into a 1 L volumetric flask, make to 1 L with distilled water and dissolve. Accurately add 787 mg of sodium sulfite and dissolve. **This is the 400 mg/L TSO_2 solution. Prepare on the day of use. Stable for 1 day at room temperature.**

Prepare the TSO_2 standard solutions as described in the table below and use directly as standards to determine A_2 in the Manual Assay Procedure For Total Sulfite (TSO_2).

CALCULATION OF TSO_2 CONTENT USING THE TSO_2 CALIBRATION CURVE:

1. TSO_2 CALIBRATION CURVE ANALYSIS:

1. Determine the absorbance (A_2) of each TSO_2 standard (STD 0-5). Subtract the absorbance of STD 0 from the absorbance of the other standards (STD 1-5), thereby obtaining $\Delta A_{TotalSO_2}$ (An example is given in Table 1).

Pipette into 13 mL polypropylene tubes	**STD 0**	**STD 1**	**STD 2**	**STD 3**	**STD 4**	**STD 5**
(TSO_2) mg/L	**0**	**40**	**80**	**160**	**320**	**400**
0.1% (w/v) citric acid (mL)	5.00	4.50	4.00	3.00	1.00	-
TSO_2 solution (mL)	-	0.50	1.00	2.00	4.00	5.00
Total Volume (mL)	5.00	5.00	5.00	5.00	5.00	5.00

2. Calculate M as follows, for each standard (STD 1-5):

$$M = \frac{TSO_2\ (mg/L)}{\Delta A_{TotalSO_2}} \qquad [(mg/L)/\Delta A_{TotalSO_2}]$$

3. Calculate the mean M as follows:

$$\text{mean } M = \frac{(M_{STD1} + M_{STD2} + M_{STD3} + M_{STD4} + M_{STD5})}{5} \ [(mg/L)/\Delta A_{TotalSO_2}]$$

Use "mean M" to calculate the TSO_2 content of the test samples in section 2 "Total Sulfite (TSO_2) Content".

Example:

Examples of the TSO_2 calibration curve calculations are given in Table 1 along with a graphical representation of a typical TSO_2 calibration curve (Figure 1).

TSO_2 standard	TSO_2 (mg/L)	A_{405}	$\Delta A_{TotalSO_2}$	M [(mg/L)/ ΔA_{TSO_2}]
STD 0	0	0.335	0.000	-
STD 1	25	0.421	0.085	293.083
STD 2	50	0.516	0.181	276.091
STD 3	100	0.717	0.382	261.986
STD 4	300	1.543	1.208	248.365
STD 5	400	1.991	1.656	241.604
mean M	-	-	-	**264.226**

Table 1. **Calculations for a typical TSO_2 calibration curve**

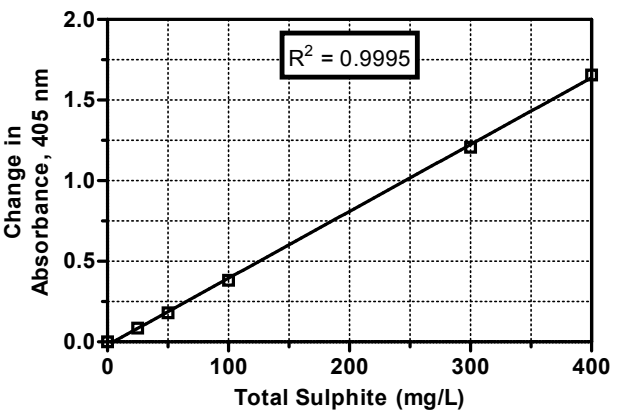

Figure 1. Calibration curve demonstrating the linearity of the colourimetric TSO_2 determination by **K-SULPH**. The sulfite standards were analysed in a cuvette with a 1.0 cm light-path.

2. TOTAL SULFITE (TSO_2) CONTENT:

Determine the $\Delta A_{TotalSO_2}$ for the sample and blank. Subtract the $\Delta A_{TotalSO_2}$ of the blank from that of the sample, thereby obtaining $\Delta A_{TotalSO_2\text{-SAMPLE}}$.

The value of $\Delta A_{TotalSO_2\text{-SAMPLE}}$ should be at least 0.100 absorbance units to achieve sufficiently accurate results.

The concentration of TSO_2 can be calculated as follows:

$$\Delta A_{TotalSO_2} = A_2 - A_1$$

$$c = \text{mean } M \times F \times \Delta A_{TotalSO_2\text{-SAMPLE}} \text{ [mg/L]}$$

where:

mean M = mean value of TSO_2 standards [(mg/L)/$\Delta A_{TotalSO_2}$]
F = dilution factor

If the sample is 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 TSO_2

$$= \frac{c_{TotalSO_2} \text{ [g/L sample solution]}}{\text{weight}_{sample} \text{ [g/L sample solution]}} \times 100 \text{ [g/100 g]}$$

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

B. MANUAL ASSAY PROCEDURE FOR FREE SULFITE (FSO_2):

Wavelength: 575 nm
Cuvette: 1 cm light path (glass or plastic)
Temperature: ~ 25°C
Final volume: 2.55 mL
Sample solution: 0.25-7.50 µg of SO_2 per cuvette (5-150 mg/L) (in 0.05 mL sample volume)
Read against air (without cuvette in the light path) or against water

Pipette into cuvettes	Sample	Standard	Blank
distilled water (at ~ 25°C)	1.00 mL	1.00 mL	1.05 mL
Free Sulfite Reagent 1	1.00 mL	1.00 mL	1.00 mL
sample	0.05 mL	-	-
standard	-	0.05 mL	-
Mix* and read absorbances of the solutions (A_1) after exactly 3 min. Then add:			
Free Sulfite Reagent 2	0.50 mL	0.50 mL	0.50 mL
Mix* and read absorbances of the solutions (A_2) after exactly 3 min.			
Mix* and read absorbances of the solutions (A_3) after exactly 3 min.			

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

PREPARATION OF THE FSO_2 SINGLE POINT STANDARD:

The FSO_2 standard is required for the calculation of FSO_2 in the test samples.
Weigh 1 g of citric acid into a 1 L volumetric flask, make to 1 L with distilled water and dissolve. Accurately add 196 mg of sodium sulfite and dissolve. **This is the 100 mg/L standard, use this directly as the single point standard in the Manual Assay Procedure for Free Sulfite (FSO_2). Prepare on the day of use. Stable for 1 day at room temperature.**

NOTE: Alternatively, a full FSO_2 calibration curve can be used and the FSO_2 content calculated as described for "Preparation of the FSO_2 Calibration Curve".

CALCULATION OF FSO_2 CONTENT USING A SINGLE POINT STANDARD:

Determine the ΔA_{FreeSO_2} for the sample, standard and blank. Subtract the ΔA_{FreeSO_2} of the blank from that of the sample and the standard, thereby obtaining $\Delta A_{FreeSO_2\text{-}SAMPLE}$ and $\Delta A_{FreeSO_2\text{-}STD}$, respectively.

The value of $\Delta A_{FreeSO_2\text{-}SAMPLE}$ and $\Delta A_{FreeSO_2\text{-}STD}$ should be at least 0.100 absorbance units to achieve sufficiently accurate results.

As the reaction between FSO_2 and the aldehyde proceeds, the equilibrium reaction between "bound" SO_2 and FSO_2 shifts towards FSO_2. With red wine this equilibrium reaction is linear and the concentration of FSO_2 can be calculated as follows:

$$\Delta A_{FreeSO_2} = (A_2 - A_1) - (A_3 - A_2)$$

$$c = \frac{\Delta A_{FreeSO_2\text{-}SAMPLE}}{\Delta A_{FreeSO_2\text{-}STD} / c_{FreeSO_2\text{-}STD}} \times F \quad [mg/L]$$

where:

$c_{FreeSO_2\text{-}STD}$ = concentration of FSO_2 standard (mg/L)
F = dilution factor

If the sample is 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 FSO_2

$$= \frac{c_{FreeSO_2}\ [g/L\ sample\ solution]}{weight_{sample}\ [g/L\ sample\ solution]} \times 100 \quad [g/100\ g]$$

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).

PREPARATION OF THE FSO_2 CALIBRATION CURVE:

Weigh 1 g of citric acid into a 1 L volumetric flask, make to 1 L with distilled water and dissolve. Accurately add 295 mg of sodium sulfite and dissolve. **This is the 150 mg/L FSO_2 solution. Prepare on the day of use. Stable for 1 day at room temperature.**

Prepare the FSO_2 standard solutions as described in the table below and use directly as standards to determine A_2 in the Manual Assay Procedure for Free Sulfite (FSO_2).

Pipette into 13 mL polypropylene tubes	**STD 0**	**STD 1**	**STD 2**	**STD 3**	**STD 4**	**STD 5**
(FSO_2) mg/L	**0**	**15**	**30**	**60**	**120**	**150**
0.1% (w/v) citric acid (mL)	5.00	4.50	4.00	3.00	1.00	-
FSO_2 solution (mL)	-	0.50	1.00	2.00	4.00	5.00
Total Volume (mL)	5.00	5.00	5.00	5.00	5.00	5.00

CALCULATION OF FSO_2 CONTENT USING THE FSO_2 CALIBRATION CURVE:

1. FSO_2 CALIBRATION CURVE ANALYSIS:

1. Determine the absorbance (A_2) of each FSO_2 standard (STD 0-5). Subtract the absorbance of STD 0 from the absorbance of the other standards (STD 1-5), thereby obtaining ΔA_{FreeSO_2} (An example is given in Table 2).

2. Calculate M as follows, for each standard (STD 1-5):

$$M = \frac{FSO_2\ (mg/L)}{\Delta A_{FreeSO_2}} \qquad [(mg/L)/\Delta A_{FreeSO_2}]$$

3. Calculate the mean M as follows:

$$\text{mean } M = \frac{(M_{STD1} + M_{STD2} + M_{STD3} + M_{STD4} + M_{STD5})}{5} \ [(mg/L)/\Delta A_{FreeSO_2}]$$

Use "mean M" to calculate the FSO_2 content of the test samples in section 2 "Free Sulfite (FSO_2) Content".

Example:

Examples of the FSO_2 calibration curve calculations are given in Table 2 along with a graphical representation of a typical FSO_2 calibration curve (Figure 2).

Table 2. **Calculations for a typical FSO_2 calibration curve**

FSO_2 standard	FSO_2 (mg/L)	A_{575}	ΔA_{FreeSO_2}	M [(mg/L)/ ΔA_{FSO_2}]
STD 0	0	0.132	0.000	-
STD 1	25	0.383	0.251	99.761
STD 2	50	0.628	0.496	100.806
STD 3	100	1.104	0.972	102.881
STD 4	125	1.364	1.232	101.469
STD 5	150	1.639	1.507	99.594
mean M	-	-	-	**100.893**

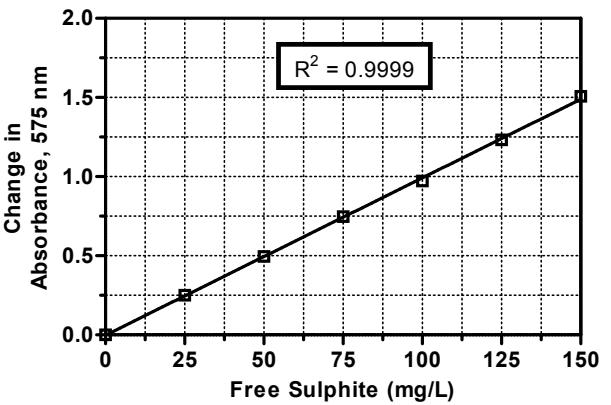

Figure 2. Calibration curve demonstrating the linearity of the colourimetric FSO_2 determination by **K-SULPH**. The sulfite standards were analysed in a cuvette with a 1.0 cm light-path.

2. FREE SULFITE (FSO_2) CONTENT:

Determine the ΔA_{FreeSO_2} for the sample and blank. Subtract the ΔA_{FreeSO_2} of the blank from that of the sample, thereby obtaining $\Delta A_{FreeSO_2\text{-SAMPLE}}$.

The value of $\Delta A_{FreeSO_2\text{-SAMPLE}}$ should be at least 0.100 absorbance units to achieve sufficiently accurate results.

As the reaction between FSO_2 and the aldehyde proceeds, the equilibrium reaction between "bound" SO_2 and FSO_2 shifts towards FSO_2. With red wine this equilibrium reaction is linear and the concentration of FSO_2 can be calculated as follows:

$$\Delta A_{FreeSO_2} = (A_2 - A_1) - (A_3 - A_2)$$

$$c = \text{mean } M \times F \times \Delta A_{FreeSO_2\text{-SAMPLE}} \text{ [mg/L]}$$

where:

mean M = mean value of FSO_2 standards $[(mg/L)/\Delta A_{FreeSO_2}]$

F = dilution factor

If the sample is 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 FSO_2

$$= \frac{c_{FreeSO_2} \text{ [g/L sample solution]}}{\text{weight}_{sample} \text{ [g/L sample solution]}} \times 100 \quad \text{[g/100 g]}$$

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

AUTO-ANALYSER ASSAY PROCEDURES:

NOTES:

1. The Auto-Analyser Assay Procedure for TSO_2 or FSO_2 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 TSO_2 or FSO_2 **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**
3. Prepare the TSO_2 or FSO_2 standards for the single point standard or full calibration curve as described for the Manual Assay Procedures for TSO_2 or FSO_2, respectively.
4. To measure FSO_2 the auto-analyser must have a slope correction capability.

A. AUTO-ANALYSER ASSAY PROCEDURE FOR TOTAL SULFITE (TSO_2):

Wavelength: 405 nm
Calculation: End-point
Temperature: ~ 25°C or 37°C
Reaction: Absorbance increase
Final volume: 0.255 mL
Linearity: 0.025-2 µg of SO_2 per cuvette (5-400 mg/L) (in 0.005 mL sample volume)

Pipette into cuvettes	Sample	Standard
Total Sulfite Reagent 1	0.100 mL	0.100 mL
distilled water	0.100 mL	0.100 mL
sample	0.005 mL	-
standard	-	0.005 mL
Read absorbances of the solutions (A_1) after exactly 3 min. Then add:		
Total Sulfite Reagent 2	0.050 mL	0.050 mL
Read absorbances of the solutions (A_2) after exactly 3 min.		

CALCULATION FORMULA FOR TSO_2 (AUTO-ANALYSER):

$A_2 - A_1$

B. AUTO-ANALYSER ASSAY PROCEDURE FOR FREE SULFITE (FSO_2):

Wavelength: 575 nm
Calculation: Kinetic
Temperature: ~ 25°C or 37°C
Reaction: Absorbance increase
Final volume: 0.255 mL
Linearity: 0.025-0.750 µg of SO_2 per cuvette (5-150 mg/L) (in 0.005 mL sample volume)

Pipette into cuvettes	Sample	Standard
Free Sulfite Reagent 1	0.100 mL	0.100 mL
distilled water	0.100 mL	0.100 mL
sample	0.005 mL	-
standard	-	0.005 mL
Read absorbances of the solutions (A_1) after exactly 3 min. Then add:		
Free Sulfite Reagent 2	0.050 mL	0.050 mL
Read absorbances of the solutions (A_2) after exactly 3 min.		
Read absorbances of the solutions (A_3) after exactly 3 min.		

CALCULATION FORMULA FOR FSO_2 (AUTO-ANALYSER):

$(A_2 - A_1) - (A_3 - A_2)$

MICROPLATE ASSAY PROCEDURES:

NOTES:

1. The Microplate Assay Procedure for TSO_2 or FSO_2 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 TSO_2 or FSO_2 **either a single point standard or a calibration curve must be performed concurrently using the same batch of reagents.**
3. Prepare the TSO_2 or FSO_2 standards for the single point standard or full calibration curve as described for the Manual Assay Procedures for TSO_2 or FSO_2, respectively.
4. Calculation of TSO_2 or FSO_2 content should be performed as described for the Manual Assay Procedures.
5. The calculation of TSO_2 or FSO_2 content, including the calibration curve analysis, can be simplified by using the Megazyme ***Mega-Calc***™, downloadable from where the product appears on the Megazyme website (www.megazyme.com).

EQUIPMENT (RECOMMENDED):

1. Disposable 96 well polystyrene clear, flat bottom microplates, e.g. Matrix Technologies Corp. cat. no. 4915 (www.matrixtechcorp.com).
2. Disposable 25 mL reagent reservoirs, e.g. Matrix Technologies Corp. cat. no. 809311 (www.matrixtechcorp.com).
3. Micro-pipettors, e.g. Gilson Pipetman® (200 µL and 1 mL) and Multichannel Micro-pipettors, e.g. Gilson Pipetman® Ultra 8-channel (1-20 µL and 20-300 µL).
4. Stop clock.
5. Analytical balance.
6. Microplate shaker, e.g. Heidolph Titramax 100 or 1000 (www.heidolph-instruments.com).
7. Microplate reader set at 405 nm or 575 mn.
8. Vortex mixer (e.g. IKA® Yellowline Test Tube Shaker TTS2).
9. Whatman No. 1 (9 cm) filter papers.

A. MICROPLATE ASSAY PROCEDURE FOR TOTAL SULFITE (TSO_2):

Wavelength: 405 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C or 37°C
Final volume: 0.255 mL
Linearity: 0.025-2.00 µg of SO_2 per cuvette (5-400 mg/L) (in 0.005 mL sample volume)
Read against air (without cuvette in the light path) or against water

Pipette into cuvettes	Sample	Standard	Blank
distilled water (at ~ 25°C)	0.100 mL	0.100 mL	0.105 mL
Total Sulfite Reagent 1	0.100 mL	0.100 mL	0.100 mL
sample	0.005 mL	-	-
standard	-	0.005 mL	-
Mix* and read absorbances of the solutions (A_1) after exactly 3 min. Then add:			
Total Sulfite Reagent 2	0.050 mL	0.050 mL	0.050 mL
Mix* and read absorbances of the solutions (A_2) after exactly 3 min.			

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

B. MICROPLATE ASSAY PROCEDURE FOR FREE SULFITE (FSO_2):

Wavelength: 575 nm
Microplate: 96-well (e.g. clear flat-bottomed, glass or plastic)
Temperature: ~ 25°C or 37°C
Final volume: 0.255 mL
Linearity: 0.025-0.750 µg of SO_2 per cuvette (5-150 mg/L) (in 0.005 mL sample volume)
Read against air (without cuvette in the light path) or against water

Pipette into cuvettes	Sample	Standard	Blank
distilled water (at ~ 25°C)	0.100 mL	0.100 mL	0.105 mL
Free Sulfite Reagent 1	0.100 mL	0.100 mL	0.100 mL
sample	0.005 mL	-	-
standard	-	0.005 mL	-
Mix* and read absorbances of the solutions (A_1) after exactly 3 min. Then add:			
Free Sulfite Reagent 2	0.050 mL	0.050 mL	0.050 mL
Mix* and read absorbances of the solutions (A_2) after exactly 3 min.			
Mix* and read absorbances of the solutions (A_3) after exactly 3 min.			

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

SAMPLE PREPARATION:

1. Sample dilution.

The amount of TSO_2 or FSO_2 present in the cuvette (i.e. in the 0.05 mL of sample being analysed) should range between 0.25 and 20 µg for TSO_2 and 0.25 and 7.5 µg for FSO_2. The sample solution must therefore be diluted sufficiently to yield a concentration between 5 and 400 mg/L for TSO_2 and 5 and 150 mg/L for FSO_2.

Dilution table (TSO_2)

Estimated concentration of TSO_2 (mg/L)	Dilution with water	Dilution factor (F)
< 400	No dilution required	1
400-4000	1 + 9	10
> 4000	1 + 99	100

Dilution table (FSO_2)

Estimated concentration of FSO_2 (mg/L)	Dilution with water	Dilution factor (F)
< 120	No dilution required	1
120-1200	1 + 9	10
> 1200	1 + 99	100

If the value of $\Delta A_{TotalSO_2}$ or ΔA_{FreeSO_2} is too low (e.g. < 0.100), weigh out more sample or dilute less strongly.

2. Sample clarification.

a. Solutions:

Carrez I solution. Dissolve 3.60 g of potassium hexacyanoferrate (II) {$K_4[Fe(CN)_6].3H_2O$} (Sigma cat. no. P9387) in 100 mL of distilled water. Store at room temperature.

Carrez II solution. Dissolve 7.20 g of zinc sulphate ($ZnSO_4.7H_2O$) (Sigma cat. no. Z4750) in 100 mL of distilled water. Store at room temperature.

Sodium hydroxide (NaOH, 100 mM). Dissolve 4 g of NaOH in 1 L of distilled water. Store at room temperature.

b. Procedure:

Pipette the liquid sample into a 100 mL volumetric flask which contains approx. 60 mL of distilled water, or weigh sufficient quantity of the sample into a 100 mL volumetric flask, and add 60 mL of distilled water. Carefully add 5 mL of Carrez I solution, 5 mL of Carrez II solution and 10 mL of NaOH solution (100 mM). Mix after each addition. Fill the volumetric flask to the mark, mix and filter.

3. 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 (such as red wine or coloured fruit juice), the pH of the solution should be increased to approx. 7.4 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. 7.4 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 polyvinylpolypyrrolidone (PVPP)/10 mL of sample. Shake the tube vigorously for 5 min and then filter through Whatman No. 1 filter paper.

(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 assay.

(g) Samples containing protein: deproteinise samples containing protein with Carrez reagents, alternatively use ice cold perchloric acid. Add 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 between 7 and 8 with 1 M KOH. Use the supernatant in the assay after appropriate dilution.

SAMPLE PREPARATION EXAMPLES:

(a) Determination of TSO_2/FSO_2 in white wine.

The TSO_2/FSO_2 concentrations of white wine can generally be determined without any sample treatment (except dilution according to the dilution table). *Typically, no dilution is required and a sample volume of 0.05 mL is satisfactory.*

(b) Determination of TSO_2/FSO_2 in red wine.

The TSO_2/FSO_2 concentrations of red wine can generally be determined without any sample treatment (except dilution according to the dilution table). *Typically, a dilution of 1:2 and sample volume of 0.05 mL are satisfactory.*

(c) Determination of TSO_2/FSO_2 in beer.

After removal of carbon dioxide by stirring with a glass rod, dilute the sample according to the dilution table and analyse. *Typically, no dilution is required and a sample volume of 0.05 mL is satisfactory.*

(d) Determination of TSO_2/FSO_2 in fruit juice.

Dilute the sample to yield a TSO_2 or FSO_2 concentration of less than 400 mg/L or 150 mg/L, respectively (see dilution table). Clear, neutral solutions can generally be determined without any sample

treatment (except dilution). Turbid liquids generally only require centrifuging or filtering before the dilution step. Coloured solutions are usually suitable for analysis after dilution to an appropriate TSO_2 or FSO_2 concentration. However, if coloured solutions require analysis undiluted, they may need decolourising as follows: adjust 25 mL of liquid sample to approx. pH 7.4 with 1 M NaOH and increase the volume to 50 mL with distilled water. Add 0.5 g of PVPP, stir for 5 min and filter through Whatman No. 1 filter paper or centrifuge. Use the clear, slightly coloured filtrate or supernatant directly in the assay. *Typically, no dilution is required and a sample volume of 0.05 mL is satisfactory.*

(e) Determination of TSO_2/FSO_2 in potatoes.

Mince approx. 50 g of potato with 50 mL of 1 M potassium phosphate buffer (pH 4) and 0.1 mL of *n*-octanol (to minimise foaming) with a household mixer for approx. 1 min. Adjust to approximately pH 4 with 2 M KOH. Quantitatively transfer the mixture to a 500 mL volumetric flask with distilled water, fill up to mark with distilled water, mix and filter through Whatman No. 1 filter paper or centrifuge. *Typically, no dilution is required and a sample volume of 0.05 mL is satisfactory.*

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