

Sulfite



interchim

BOEHRINGER MANNHEIM / R-BIOPHARM
Enzymatic BioAnalysis / Food Analysis

UV-method

for the determination of sulfurous acid ("total SO₂") in food-stuffs and other materials

Cat. No. 10 725 854 035

Test-Combination for 31 determinations

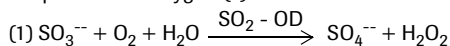
For use in *in vitro* only

Store at 2-8°C

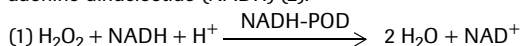
For recommendations for methods and standardized procedures see references (2)

Principle (Ref. 1)

Sulfite (sulfurous acid) is oxidized to sulfate by sulfite oxidase (SO₂-OD) in the presence of oxygen (1).



The hydrogen peroxide formed in this reaction is reduced by the enzyme NADH-peroxidase (NADH-POD) in the presence of reduced nicotinamide-adenine dinucleotide (NADH) (2).



The amount of NADH oxidized in reaction (2) is equivalent to the amount of sulfite or to aldehyde chemically-bound sulfite. NADH is determined by means of its light absorbance at 334, 340 or 365 nm.

(Note: Hydrogen peroxide formed as an intermediate product does not react with the residual sulfite.)

The Test-Combination contains

1. Bottle 1 with approx. 30 ml solution, consisting of: triethanolamine buffer, pH approx. 8.0
2. Bottle 2 with approx. 30 tablets; each tablet contains: NADH, approx. 0.4 mg
3. Bottle 3 with approx. 0.3 ml suspension, consisting of: NADH-POD, approx. 3 U
4. Bottle 4 with approx. 1.6 ml suspension, consisting of: SO₂-OD, approx. 4 U¹

Preparation of solutions

1. Use contents of bottle 1 undiluted.
2. Dissolve **one tablet** of bottle 2 with **one ml** solution of bottle 1 in a beaker or in a centrifuge tube for each assay (blank and samples) depending on the number of determinations. Use forceps for taking the tablets out of bottle 2. This results in reaction mixture 2*.
3. Use contents of bottle 3 undiluted.
4. Use contents of bottle 4 undiluted.

Stability of reagents

Solution 1 is stable at 2-8°C (see pack label).

Bring solution 1 to 20-25°C before use.

The contents of bottle 2 are stable at 2-8°C (see pack label).

Reaction mixture 2 is stable for one week at 2-8°C.

Bring reaction mixture 2 to 20-25°C before use.

The contents of bottles 3 and 4 are stable at 2-8°C (see pack label).

Procedure

Wavelength²: 340 nm, Hg 365 nm or Hg 334 nm
Glass cuvette³: 1.00 cm light path
Temperature: 20-25°C
Final volume: 3.060 ml
Read against air (without a cuvette in the light path) or against water
Sample solution: 0.6-30 µg sulfite/assay⁴ (as SO₂; in 0.100 - 2.000 ml sample volume)

Important note

Use only freshly redist. water for the assay or treat demineralized water with activated charcoal (e.g. 1 g/100 ml): mix charcoal into water while stirring. Filter after approx. 3 min. Bentonite may also be used.

1 Activity, measured as O₂-consumption, 1 µmol/min.

2 The absorption maximum of NADH is at 340 nm. On spectrophotometers, measurements are taken at the absorption maximum; if spectralline photometers equipped with a mercury vapor lamp are used, measurements are taken at a wavelength of 365 nm or 334 nm.

3 If desired, disposable cuvettes may be used instead of glass cuvettes.

4 See instructions for performance of assay.

5 Available from Roche Applied Science, Cat. No. 236 314

6 Available from Roche Applied Science, Cat. No. 736 619

Pipette into cuvettes	Blank	Sample
reaction mixture 2*	1.000 ml	1.000 ml
sample solution**	-	0.100 ml
redist. water	2.000 ml	1.900 ml
suspension 3	0.010 ml	0.010 ml
Mix***, read absorbances of the solutions after approx. 5 min (A ₁). Start the reaction by addition of:		
suspension 4	0.050 ml	0.050 ml
Mix***, wait for the completion of the reaction (approx. 30 min), read absorbances of the solutions (A ₂). If the reaction has not stopped after 30 min, continue to read the absorbances at 5 min intervals until the absorbances decrease constantly over 5 min.		

* For simplification of the assay performance it is also possible to pipette directly 1.000 ml of solution 1 into the cuvette and to add 1 tablet from bottle 2. After dissolution of the tablet with the aid of a spatula continue working as described in the procedure. The difference in volume of ca. 1% (increase of volume by 1 tablet per 3.060 ml assay volume) has to be taken into account in the calculation by multiplication of the result with 1.01.

** Rinse the enzyme pipette or the pipette tip of the piston pipette with sample solution before dispensing the sample solution.

*** For example, with a plastic spatula or by gentle swirling after closing the cuvette with Parafilm (trademark of the American Can Company, Greenwich, Ct., USA)

If the absorbance at A₂ decreases constantly, extrapolate the absorbances to the time of the addition of suspension 4 (sulfite oxidase).

Determine the absorbance differences (A₁-A₂) for both, blank and sample. Subtract the absorbance difference of the blank from the absorbance difference of the sample.

$$\Delta A = (A_1 - A_2)_{\text{sample}} - (A_1 - A_2)_{\text{blank}}$$

The measured absorbance differences should, as a rule, be at least 0.100 absorbance units to achieve sufficiently precise results (see "Instructions for performance of assay" and "Sensitivity and detection limit", pt.4).

Occasionally a negative value with (A₁-A₂)_{blank} is obtained. This value is then to be added to (A₁-A₂)_{sample} according to the calculation formula.

If the absorbance difference of the sample (ΔA_{sample}) is higher than 1.000 (measured at 340 nm, or Hg 334 nm, respectively) or 0.500 (measured at Hg 365 nm), the concentration of sulfite in the sample solution is too high. The sample is to be diluted according to the dilution table in that case.

Calculation

According to the general equation for calculating the concentration:

$$c = \frac{V \times MW}{\epsilon \times d \times v \times 1000} \times \Delta A \text{ [g/l]}$$

V = final volume [ml]

v = sample volume [ml]

MW = molecular weight of the substance to be assayed [g/mol]

d = light path [cm]

ε = extinction coefficient of NADH at:
340 nm = 6.3 [l × mmol⁻¹ × cm⁻¹]
Hg 365 nm = 3.4 [l × mmol⁻¹ × cm⁻¹]
Hg 334 nm = 6.18 [l × mmol⁻¹ × cm⁻¹]

It follows for sulfite (as SO₂):

$$c = \frac{3.060 \times 64.06}{\epsilon \times 1.00 \times 0.100 \times 1000} \times \Delta A = \frac{1.960}{\epsilon} \times \Delta A \text{ [g SO}_2\text{/l sample solution]}$$

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

When analyzing solid and semi-solid samples which are weighed out for sample preparation, the result is to be calculated from the amount weighed:

$$\text{Content}_{\text{sulfite}} = \frac{c_{\text{sulfite}} [\text{g/l sample solution}]}{\text{weight}_{\text{sample}} \text{ in g/l sample solution}} \times 100 [\text{g}/100 \text{ g}]$$

1. Instructions for performance of assay

The sulfite content present in the assay has to be between 0.6 µg and 30 µg. In order to obtain a sufficient absorbance difference, the sample solution is diluted to yield a sulfite concentration between 0.03 and 0.3 g/l.

Dilution table

Estimated amount of sulfite per liter	Dilution with water	Dilution factor F
< 0.3 g	-	1
0.3-3.0 g	1 + 9	10
3.0-30 g	1 + 99	100
> 30 g	1 + 999	1000

If the measured absorbance difference (ΔA) is too low (e.g. < 0.100), the sample solution should be prepared again (weigh out more sample or dilute less strongly) or the sample volume to be pipetted into the cuvette can be increased up to 2.000 ml. The volume of water added must then be reduced so as to obtain the same final volume in the assays for sample and blank. The new sample volume v must be taken into account in the calculation.

2. Technical instructions

When determining sulfite in trace-level compound analysis some points have to be considered in order to obtain satisfactory results:

2.1 Ingredients of the sample (e.g. of spices or tea) may cause smaller or larger sample-dependent creep reactions. If these are not taken into consideration (or in a wrong way) the results will be wrong especially in the case of low sulfite contents.

Therefore, it is recommended to read A_2 10 min after the addition of suspension 4 (sulfite oxidase) and to repeat the measurements at 10 min intervals until the absorbance decreases constantly over 10 min. The absorbance has to be extrapolated to the time of the addition of suspension 4 (sulfite oxidase).

2.2 Sulfurous acid (SO_2) is volatile, reactive and easily oxidized. These characteristics of the analyte have to be considered when assessing data from analysis or when comparing data from different methodologies.

3. Specificity (Ref. 1)

Sulfite oxidase reacts with sulfite, with isothiocyanates and their glycosides, so that in the analysis e.g. of mustard, leek, garlic, horse radish and onions too high results are obtained.

Organic sulfonic acid-compounds cause a slight creep reaction.

Sulfides, thiosulfate, sulfate and organic sulfinic acid-compounds do not react under assay conditions.

Sodium sulfite, as well as sodium and potassium disulfite (pyrosulfite, metabisulfite) absorb moisture and are oxidized easily (formation of sulfate). Aqueous solutions are instable. Therefore, results of < 100 % have to be expected in analysis. If demineralized water is used for the preparation of the (di-) sulfite solutions, greater losses of sulfite are observed.

4. Sensitivity and detection limit (Ref. 1.4)

The smallest differentiating absorbance for the procedure is 0.005 absorbance units. This corresponds to a maximum sample volume $v = 2.000$ ml and measurement at 340 nm of a sulfite concentration of 0.1 mg/l sample solution (if $v = 0.100$ ml, this corresponds to approx. 1.5 mg/l sample solution).

The detection limit of 0.3 mg/l is derived from the absorbance difference of 0.020 (as measured at 340 nm) and a maximum sample volume $v = 2.000$ ml.

5. Linearity

Linearity of the determination exists from 0.6 µg sulfite/assay (0.3 mg sulfite/l sample solution; sample volume $v = 2.000$ ml) to 30 µg sulfite/ assay (0.3 g sulfite/l sample solution; sample volume $v = 0.100$ ml).

6. Precision

In a double determination using one sample solution, a difference of 0.005 to 0.010 absorbance units may occur. With a sample volume of $v = 0.100$ ml and measurement at 340 nm, this corresponds to a sulfite concentration of approx. 1.5-3 mg/l. (If the sample is diluted during sample preparation, the

result has to be multiplied by the dilution factor F . If the sample is weighed in for sample preparation, e.g. using 1 g sample/100 ml = 10 g/l, a difference of 0.015-0.03 g/100 g can be expected.)

The following data have been published in the literature:

5 µg/assay	$n = 15$	$CV = 5.71\%$ (in series)	
10 µg/assay	$n = 15$	$CV = 2.79\%$ (in series)	
20 µg/assay	$n = 15$	$CV = 3.21\%$ (in series)	(Ref. 1.1)

Sultana:

$x = 260$ mg/kg	$r = 45$ mg/kg	$s_{(r)} = \pm 16$ mg/kg
	$R = 29$ mg/kg	$s_{(R)} = \pm 46$ mg/kg

Beer:

$x = 4.9$ mg/l	$r = 0.8$ mg/l	$s_{(r)} = \pm 0.3$ mg/l
	$R = 1.6$ mg/l	$s_{(R)} = \pm 0.6$ mg/l

(Ref. 2.1)

7. Interference

Sulfite oxidase is inhibited by L-ascorbic acid (> 20 µg L-ascorbic acid/assay; reaction time: approx. 40 min), but the sulfite content in the sample is completely found.

The presence of high L-ascorbic acid concentrations in the assay (> 50 µg L-ascorbic acid/assay) does not only cause inhibition of the enzyme but also causes a low recovery of sulfite (approx. 80%) because of the reaction with intermediary formed H_2O_2 . Therefore, L-ascorbic acid-containing samples should be treated with ascorbate oxidase (see "Determination of sulfite in fruit juices", pt. 11).

8. Recognizing interference during the assay procedure

8.1 If the conversion of sulfite has been completed according to the time given under "Procedure", it can be concluded in general that no interference has occurred.

8.2 On completion of the reaction, the determination can be restarted by adding sulfite (qualitative or quantitative): if the absorbance is altered subsequent to the addition of the standard material, this is also an indication that no interference has occurred.

8.3 Operator error or interference of the determination through the presence of substances contained in the sample can be recognized by carrying out a double determination using two different sample volumes (e.g. 0.100 ml and 0.200 ml): the measured differences in absorbance should be proportional to the sample volumes used.

When analyzing solid samples, it is recommended that different quantities (e.g. 1 g and 2 g) be weighed into 100 ml volumetric flasks. The absorbance differences measured and the weights of sample used should be proportional for identical sample volumes.

8.4 Possible interference caused by substances contained in the sample can be recognized by using an internal standard as a control: in addition to the sample, blank and standard determinations, a further determination should be carried out with sample and assay control solution in the same assay. The recovery can then be calculated from the absorbance differences measured.

8.5 Possible losses during the determination can be recognized by carrying out recovery tests: the sample should be prepared and analyzed with and without added standard material. The additive should be recovered quantitatively within the error range of the method.

9. Reagent hazard

The reagents used in the determination of sulfite are not hazardous materials in the sense of the Hazardous Substances Regulations, the Chemicals Law or EC Regulation 67/548/EEC and subsequent alteration, supplementation and adaptation guidelines. However, the general safety measures that apply to all chemical substances should be adhered to.

After use, the reagents can be disposed of with laboratory waste, but local regulations must always be observed. Packaging material can be disposed of in waste destined for recycling.

10. General information on sample preparation

In carrying out the assay:

Use **clear, colorless and practically neutral liquid samples** directly, or after dilution according to the dilution table, and of a volume up to 2.000 ml; Filter or centrifuge **turbid solutions**;

Degas **samples containing carbon dioxide** (e.g. by filtration);

Adjust **acid samples** to approx. pH 8 by adding sodium or potassium hydroxide solution;

Adjust **acid and weakly colored samples** to approx. pH 8 by adding sodium or potassium hydroxide solution and incubate for approx. 15 min;

Treat "strongly colored" samples that are used undiluted or with a higher sample volume with polyvinylpyrrolidone, PVPP;

Crush or homogenize **solid or semi-solid samples**, extract with water or dissolve in water and filter if necessary;

Extract **samples containing fat** with hot water in a closed bottle (extraction temperature should be above the melting point of the fat involved). Cool to allow the fat to separate, make up to the mark, place the volumetric flask in an ice bath for 15 min and filter or centrifuge.

Use sample solutions for the assay as soon as possible.

11. Application examples

Determination of sulfite (total SO₂) in white wine

Use white wine directly for the assay with a sample volume of $v = 0.100$ ml.

Determination of sulfite in red wine

Adjust 25 ml red wine to pH 7.5-8.0 with sodium hydroxide (2 M) and dilute to 50 ml with redist. water in a volumetric flask (dilution factor $F = 2$). Incubate for approx. 10 min at 20-25°C Use 0.100 ml for the assay.

Determination of sulfite in spirits

Use brandy undiluted for the assay (sample volume $v = 0.500$ ml).

Determination of sulfite in beer

Bring beer sample to 20-25°C, open bottle and filter beer sample immediately. Add 0.7 g bentonite to 10 ml beer in a 50 ml beaker, stir for 1 min and filter. Use 1.000 ml filtrate for assay.

The occurring "creep reaction" should be taken into account in the calculation with $\Delta mA/min < 2$.

Determination of sulfite in absinth and cola beverages

Use samples directly for the assay, increase the sample volume up to 0.5 ml, if necessary. Occurring "creep reactions" should be taken into account by extrapolation.

Determination of sulfite in fruit juices

Centrifuge turbid juices (approx. 4000 rpm). For removal of L-ascorbic acid (if at all necessary: > 100 mg L-ascorbic acid/l sample), adjust 2.0 ml of the clear juice to pH 5-6 with sodium hydroxide (2 M; measure the volume of the added NaOH and take into account in the calculation), add approx. 20 U ascorbate oxidase⁵ (approx. 0.1 mg lyophilizate), mix and incubate for 10 min. The L-ascorbic acid can also be removed by stirring 2.0 ml juice with an ascorbate oxidase spatula⁶ for approx. 3 min. Then adjust the sample to pH 7.5-8.0 with sodium hydroxide (2 M) (measure the volume of the added NaOH and take into account in the calculation), add 0.1 g polyvinylpyrrolidone (PVPP) (only necessary for colored juices), stir for 1 min and filter. Use 0.100 ml (up to 1.000 ml, if necessary) of the largely decolorized sample for the assay.

Determination of sulfite in jam

Homogenize approx. 100 g jam in a homogenizer for 30 s. Accurately weigh approx. 5 g of the homogenous sample into a 50 ml volumetric flask and add 40 ml redist. water. Close the volumetric flask and incubate at 60°C for 5 min. Shake from time to time. After cooling to 20-25°C, fill up to the mark with redist. water, mix and filter. Use the clear solution diluted according to the dilution table, if necessary, for the assay.

Determination of sulfite in potato products

Mince and homogenize dried potato products using a mortar or mixer. Accurately weigh approx. 5 g of minced and ground potato chips (or approx. 2 g ground dried potato material intended for production of potato dumplings) into a 100 ml volumetric flask and add 80 ml hot (65°C) redist. water. Close the volumetric flask and shake rigorously for 5 min or stir with magnetic stirrer, afterwards let it stand respectively for 15 min. After cooling to 20-25°C, fill up to the mark with redist. water, mix and centrifuge (10 min at 4000 rpm). Use the clear solution with a sample volume of up to 0.500 ml or diluted according to the dilution table, if necessary, for the assay.

Note:

Ingredients of potato products, e.g. (poly-) phenols, can cause pre-reactions. Therefore, A₁ is to be read 20 min after addition of the sample solution, A₂ 30 min after addition of sulfite oxidase and A₃ after further 30 min.

The absorbance difference is calculated from: $\Delta A = (A_1 - A_2) - (A_2 - A_3)$.

Determination of sulfite in fruit and vegetable products

It is recommended to proceed as stated under "Determination of sulfite in potato products".

Determination of sulfite in spices and coffee products

Mince and homogenize the spice sample using a mortar or mixer. Accurately weigh approx. 100 mg sample into a 50 ml volumetric flask and add 30 ml redist. water. Close the volumetric flask and incubate at approx. 60°C for 5 min. Shake from time to time. After cooling to 20-25°C, fill up to the mark with redist. water, mix and filter. Use the clear solution diluted according to the dilution table, if necessary, for the assay.

Determination of sulfite in hop products

Mince and homogenize the sample with a suitable homogenizer (it should be possible to cool the sample material; too much heating of the sample should be avoided: temperature not higher than approx. 30°C). Accurately weigh approx. 3 g of the homogenized sample material into a 100 ml volumetric flask, add approx. 60 ml redist. water and mix. Incubate at 20-25°C (not higher than 25°C) for 10 min, fill up to the mark with redist. water, mix and filter. Discard the first few ml of the filtrate; add 0.1 g EDTA to 10 ml of the filtrate, stir for 2 min, and filter, if necessary. Use the clear filtrate for the assay.

12. Further applications

The method may also be used in the examination of tap water, sewage water, air and smoke gas.

12.1 Determination of sulfite in exhaust gas, smoke gas and air (Ref. 3.1)

Take the sample in a suitable manner and wash it in two gas wash bottles fitted together, filled with 80 ml sodium hydroxide (1 M) each (3 l/min, for approx. 10 min). Use the sample from the first gas wash bottle immediately for the assay, diluted according to the dilution table, if necessary.

For calculation of the result, temperature, air pressure, concentration of oxygen, flow speed, washing time, and also other ingredients of the gas, if necessary, have to be taken into account.

12.2 Determination of sulfite in cellulose waste water

Homogenize the sample (mixer, homogenizer, etc.). Accurately weigh approx. 5 g of the sample material into a 100 ml volumetric flask, add 60 ml redist. water and mix. Add 100 mg EDTA and incubate at 20-25°C (not higher than 25°C) for 10 min. Adjust to pH 7.5-9.5 with sodium hydroxide (2 M), fill up to the mark with redist. water, mix and filter. Add 1 g polyvinylpyrrolidone (PVPP) to 10 ml of the filtrate and stir with a magnetic stirrer for 2 min. Filter, discard the first few ml of the filtrate, and use the filtrate for the assay.

If the sample contains a high amount of heavy metals (e.g. > 100 mg lead/100 g sample material) a higher quantity of EDTA is to be added.

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Sulfite assay control solution

The assay control solution serves as a control for the enzymatic determination of sulfite in foodstuffs and other materials.

Reagents

Sodium sulfite, anhydrous, AR grade (Na_2SO_3 ; M = 126.04 g/mol; contains 50.8 % SO_2), or

Sodium disulfite (sodium metabisulfite, sodium pyrosulfite), AR grade ($\text{Na}_2\text{S}_2\text{O}_5$; M = 190.10 g/mol; contains 67.4 % SO_2), or

Potassium disulfite, AR grade ($\text{K}_2\text{S}_2\text{O}_5$; M = 222.33 g/mol; contains 57.6 % SO_2)

Preparation of the assay control solution

Accurately weigh approx. 60 mg sodium sulfite, resp. 45 mg sodium disulfite, resp. 50 mg potassium disulfite, to the nearest 0.1 mg into a 100 ml volumetric flask, fill up to the mark with redist. water, and mix thoroughly (corresponds approx. 0.3 g sulfite/l).

Prepare assay control solution with redist. tap water immediately before use (see "Important note").

Application:

1. Addition of sulfite assay control solution to the assay mixture:

Instead of sample solution the assay control solution is used for the assay. (For the calculation of results use the molecular weight of the standard material weighed in for analysis.)

The measurement of the assay control solutions is not necessary for calculating the results.

2. Restart of reaction, quantitatively:

After completion of the reaction with sample solution and measuring A_2 , add 0.050 ml assay control solution to the assay mixture. Read absorbance A_3 after the end of the reaction (approx. 30 min). Calculate the concentration from the difference ($A_2 - A_3$) according to the general equation for calculating the concentration. The altered total volume must be taken into account. Because of the dilution of the assay mixture by addition of the assay control solution, the result differs insignificantly from the result got according to pt. 1.

3. Internal standard:

The assay control solution can be used as internal standard in order to check the determination for correct performance (gross errors) and to see whether the sample solution is free from interfering substances:

Pipette into cuvettes	Blank	Sample	Standard	Sample + Standard
reaction mixture 2	1.000 ml	1.000 ml	1.000 ml	1.000 ml
sample solution	-	0.100 ml	-	0.050 ml
assay control sln.	-	-	0.100 ml	0.050 ml
redist. water	2.000 ml	1.900 ml	1.900 ml	1.900 ml
suspension 3	0.010 ml	0.010 ml	0.010 ml	0.010 ml

Mix, and read absorbances of the solutions (A_1) after approx. 5 min. Continue as described in the pipetting scheme under "Procedure". Follow the instructions given under "Instructions for performance of assay" and the footnotes.

The recovery of the standard is calculated according to the following formula:

$$\text{recovery} = \frac{2 \times \Delta A_{\text{sample + standard}} - \Delta A_{\text{sample}}}{\Delta A_{\text{standard}}} \times 100 [\%]$$

4. Recovery experiments with original samples:

For checking sample preparation and assay, recovery experiments may be carried out. For this, either the a. m. assay control solutions are used or another assay control solution with a suitable concentration is prepared.

The original sample is measured with and without sulfite. The amount of added sulfite

- is either the same as expected to be present in the original sample,
- or corresponds to that amount of sulfite which is allowed to be contained in the sample e.g. according to standards or other regulations.

The instability of aqueous solutions of sulfite/the ease of oxidation of sulfite have to be considered when evaluating results from recovery experiments.

When calculating the recovery of the added standard material, the following factors have to be used:

Calculation of sulfite as sodium sulfite:

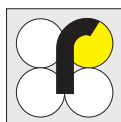
$$F = 126.04 : 64.06 = 1.968$$

Calculation of sulfite as sodium disulfite:

$$F = (190.10/2) : 64.06 = 1.484$$

Calculation of sulfite as potassium disulfite:

$$F = (222.33/2) : 64.06 = 1.735$$



211 bis av. J.F. Kennedy - B.P. N° 1140
03103 Montluçon Cedex - France
☎(33) (0)4 70 03 88 55 (+)
Fax (33) (0)4 70 03 82 60
interbiotech@interchim.com

