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A New Enzymatic Method for Determination of Sulphite in Food

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ABSTRACT

A novel enzymatic method for determining sulphite is described. Using the enzyme sulphite oxidase (EC 1.8.3.1) isolated from chicken liver, sulphite is oxidised to sulphate and hydrogen peroxide is formed. This is allowed to react with reduced nicotinamide-adenine dinucleotide (NADH) in the presence of NADH-peroxidase from microorganisms (EC 1.11.1.1). The decrease of NADH, which is proportional to the concentration of sulphite, is measured photometrically.

In spite of doubts about determining sulphite quantitatively according to this principle, the reaction conditions could be optimised: sulphite is oxidised in triethanolamine buffer (TEA) at pH 8·0 with 40 mU/ml sulphite oxidase and 47 mU/ml NADH-peroxidase quantitatively within a period of 20 min. The precision of measurement is very high in the range 13–205 µmol sulphite/litre test solution. The coefficient of variation is found to be CV = 0·67% in a solution of 67 µmol SO_2/litre.

The accuracy of the enzymatic measurements in pure aqueous sulphite solutions is confirmed by comparison with chemical determination. The correlation with the iodimetric method is R = 0·995.

High specificity for sulphite is found when applying the enzymatic test described here. Carbonyl/sulphite addition compounds (e.g. those occurring in wine) are converted to sulphate and the free carbonyl compound. The majority of sulphur-containing compounds do not react in the test. Only glycosides of isothiocyanates (e.g. in mustard) are oxidised like sulphite, though more slowly.

The majority of substances occurring in food do not interfere

significantly with the test. Ascorbic acid influences the determination if it is present in high concentrations. Therefore, it should be removed from the sample before measuring sulphite.

The method has been proven in use for a large number of foodstuffs. By using the method described here, sulphite can be determined in food rapidly and with high reliability.

ANALYTICAL PROBLEMS

The enzymatic determination of sulphite follows the reaction scheme below:

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

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

Sulphite ions are oxidised to sulphate ions by oxygen in the presence of the sulphite oxidase, thereby forming hydrogen peroxide. This is reduced to water by the reduced nicotinamide-adenine dinucleotide (NADH) in the presence of NADH-peroxidase. In this reaction, NAD^+ is formed (and NADH is consumed) in amounts proportional to the sulphite concentration. The consumption of NADH can be measured photometrically at 339 nm, 334 nm (Hg) and 365 nm (Hg).

To achieve a quantitative SO_2 reaction, three conditions must be fulfilled:

(1) reaction in an alkaline medium causing the chemical reaction to proceed slowly;
(2) high affinity of the sulphite oxidase towards sulphite given by the Michaelis constant $K_M = 2{\cdot}4 \times 10^{-2}$ M (pH 8·5, TRIS buffer), and high amounts of enzyme;
(3) rapid removal of H_2O_2 in reaction 2, e.g. by reaction with chromogenic substrates in the presence of peroxidases.

The latter condition can only be fulfilled with difficulty; the usual enzymatic methods for converting H_2O_2 fail because the rate of reaction of peroxidase with H_2O_2 is rather low. Other potential problems are:

Reaction of SO_3^{2-} with H_2O_2.
Inhibition of peroxidases by SO_3^{2-}.
Reaction of SO_3^{2-} with chromogenic dyes in the reduced form.
Destruction of oxidised chromogenic dyes by SO_3^{2-}.

It is fortunate that NADH-peroxidase (EC 1.11.1.1) possesses a high affinity towards H_2O_2 ($K_m = 2{\cdot}8 \times 10^{-5}$ mol/litre, TRIS buffer 0·2 mol/litre, pH = 6·0). This enzyme is not inhibited by excess sulphite, thus the essential conditions for an enzymatic sulphite assay were satisfied.

OPTIMISATION OF THE SULPHITE ASSAY

Optimum pH for the test system

The reaction of sulphite with the sulphite oxidase is best at pH 8.4. The reaction of the H_2O_2 formed with the NADH-peroxidase is best at pH ca. 6·0. The reaction of the combined reagents proceeds most satisfactorily at pH 8·2. Because of the stability of NADH, pH 8·0 was chosen for the assay.

Buffer substances

Phosphate buffers using substances which may be used in buffers at pH 8·0 include sodium and potassium phosphates, glycine, HEPES (4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid), MES (4-morpholine-ethane sulphonic acid), TRIS (tris-(hydroxymethyl)aminoethane) and TEA. The potential problems associated with these are listed below:

K-phosphate, Na-phosphate: Na^+ activates contaminating NADH-oxidases. K^+: inhibition of the NADH-peroxidase.
Pyrophosphate: strong inhibition of NADH-peroxidase and sulphite oxidase.
Glycine buffer: slight inhibition of the enzymes by glycylglycine and glycine.
HEPES and MES buffer: slight inhibition of the enzymes and colour changing by HEPES and MES-buffer substances.
TRIS buffer: no known problems.

TEA buffer was chosen for the analyses.

Buffer concentration

The test is operable in buffer solutions at concentrations in the range 0·1–0·25M.

Enzyme requirements

NADH-peroxidase: 17 mU*/ml test solution gives a 96 % conversion of sulphite after 30 min reaction time. At a higher concentration (80 mU/ml test solution) interfering reactions by NADH-oxidases took place.

Sulphite oxidase: 20 mU/ml test solution gives a 95 % conversion of sulphite after 40 min reaction time. 40 mU/ml test solution gives a 99 % conversion of sulphite after 15 min reaction time.

Ratio NADH-peroxidase/sulphite oxidase: 1·6–2·5 with sulphite oxidase: ca. 30 mU/ml test solution.

Reaction velocity and kinetics

Under the reaction conditions specified sulphite at a concentration of 200 μM test solution ($\hat{=}$ 40 μg SO_2/cuvette), gives rise to a qualitative conversion of NADH within 20 min.

Contaminating NADH-oxidase leads to a creeping reaction at a rate of 0·5–5·0 ΔmA/min (where A is absorbance at = 365 nm (Hg)).

REAGENTS AND APPARATUS

The reagents used were: sodium sulphite, a.r. (Na_2SO_3); sodium hydrogencarbonate, a.r. ($NaHCO_3$): sodium hydroxide (0·1M NaOH); triethanolamine hydrochloride (TEA); β-nicotinamide-adenine dinucleotide, reduced (NADH); NADH-peroxidase; sulphite oxidase. (The biochemical reagents were from Boehringer Mannheim, West Germany, the chemical reagents from Merck, Darmstadt, West Germany.)

The apparatus used was a spectrophotometer or a spectrum-line photometer with mercury lamp for measuring at 339 nm, 334 nm (Hg) and 365 nm (Hg).

PREPARATION OF SOLUTIONS

The solutions prepared (for about 40 determinations) were:

I. TEA buffer (0·6 mol/litre, = 5·57 g TEA/50 ml, adjusted to pH 8·0 with NaOH (0·1M)).

* 1U = The amount of sulphite oxidase which consumes 1 μmol O_2 per min under test conditions (TEA buffer, 0·2M, pH 8·0, 25 °C).

II. NADH solution (ca. 7 mM = 25 mg NADH-Na_2 and 50 mg $NaHCO_3$ dissolved in 5·0 ml water).
III. NADH-peroxidase suspension (ca. $4{\cdot}5 \times 10^4$ U/litre—For application dilute 0·1 ml of the enzyme suspension with 0·2 ml aqueous ammonium sulphate solution (3·0M = 396 g/litre, pH ca. 7).
IV. Sulphite oxidase suspension (ca. 5000 U/litre).—For application dilute 1·0 ml of the enzyme suspension with 1·0 ml aqueous ammonium sulphate solution (3·0M = 396 g/litre, pH ca. 6).

Sample solution: ca. 60–600 mg Na_2SO_3/litre (≙ ca. 30–300 mg SO_2/litre, ca. 0·47–4·7 mM).

MEASUREMENT PROCEDURE

The absorbances were measured at 339 nm, 334 nm (Hg) or 365 nm (Hg), using glass cuvettes or disposable cuvettes of 1 cm path length. The temperature was ca. 25 °C and the final volume was 3·16 ml. Measurement was made against air. Details are given in Table 1.

The next step is to determine the absorbance differences ($A_1 - A_2$) for

TABLE 1

Pipette into cuvette		*Reagent blank (ml)*	*Sample (ml)*	*Concentration in assay*
TEA buffer	(I)	1·00	1·00	TEA, 190 mM
NADH solution	(II)	0·10	0·10	NADH, 220 μM
NADH-peroxidase	(III)	0·01	0·01	NADH-peroxidase, 47 U/litre
Sample solution		—	0·10	SO_2, up to 150 μM
Water		2·00	1·90	
Mix. After about 5 min measure absorbance of the solution (A_1); start reaction by addition of:				
Sulphite oxidase	(IV)	0·05	0·05	Sulphite oxidase, 390 U*/litre
Mix. On completion of the reaction (ca. 20–30 min), measure the absorbance of the solution (A_2). If the reaction has not stopped after 30 min, continue to measure the absorbance at 2 min intervals until the absorbance decreases constantly over a period of 2 min.				

From the constant decrease in absorbance A_2, the absorbance is extrapolated to the moment of the addition of suspension (IV), i.e. zero reaction time.

both blank and sample, and then subtract the absorbance difference of the blank (ΔA_B) from the absorbance difference of the sample (ΔA_S)

$$\Delta A_{\text{sulphite}} = \Delta A_S - \Delta A_B$$

Usually the sulphite reaction is accompanied by a creeping reaction (because of contaminating NADH-oxidase). It can be ignored if the absorbances A_2 of the sample and the blank are read simultaneously, because the creeping reaction of the sample is normally the same as that of the blank.

CALCULATION

The concentration of sulphite can be calculated according to the general formula for nicotinamide-coenzyme-dependent reactions. Under the conditions of the assay the concentration of sulphite is:

$$C_{\text{sulphite, SO}_2}(\text{g/litre}) = \frac{0{\cdot}2024}{\varepsilon} \times \Delta A$$

ε at 365 nm (Hg) $= 0{\cdot}34\ \text{mM}^{-1}\,\text{mm}^{-1}$
ε at 339 nm $= 0{\cdot}63\ \text{mM}^{-1}\,\text{mm}^{-1}$
ε at 334 nm (Hg) $= 0{\cdot}618\ \text{mM}^{-1}\,\text{mm}^{-1}$.

STATISTICAL DATA

The stability of the diluted solutions of SO_2 is given in Table 2.

The coefficients of variance for repeated determinations on sulphite solutions are given in Table 3.

TABLE 2

Stability of Diluted Solutions of SO_2 (in form of Na_2SO_3). Measured concentrations are shown as % SO_2

	Freshly prepared	*After 30 min (4°C)*	*Loss*	*After 30 min (25°C)*	*Loss*	*After 2 h (25°C)*	*Loss*
Na_2SO_3 (0·2% SO_2)	0·190%	0·188%	1%	0·186%	2%	0·135%	30%

TABLE 3
Results of Repeated Determinations on Standard Sulphate Solution as a Function of Concentration and Time for which Solutions had been Allowed to Stand

	Number of determinations	*μg SO_2/cuvette*	*Standard deviation μg SO_2/cuvette*	*CV*[b] (%)
Na_2SO_3, 0·48 g/litre, freshly prepared	8	24·3	0·36	1·5
Na_2SO_3, 0·295 g/litre, freshly prepared	15	15	0·10	0·67
Na_2SO_3, 0·48 g/litre, after 30 min, 4 °C	8	24·3	0·16	0·66
Na_2SO_3, 0·098 g/litre,[a] after 2 h	15	5	0·29	5·71
Na_2SO_3, 0·197 g/litre, after 2 h	15	10	0·28	2·79
Na_2SO_3, 0·394 g/litre, after 2 h	15	20	0·64	3·21

[a] Small differences of absorption
[b] CV = coefficient of variation.

Linearity

Calibration data are linear in the range 15–205 μmol SO_2/litre of test solution ($\hat{=}$ 3–40 μg sulphite/cuvette; $\Delta A_{365\,nm} = 0{\cdot}04$–$0{\cdot}62$).

In the range 2–38 μg SO_2/cuvette the correlation coefficient for values of sulphite, enzymatically measured, is 1·000.

SPECIFICITY OF THE ENZYMATIC ASSAY

The method described here is specific for sulphite. Sulphur-containing compounds such as sulphate, sulphides and thiosulphates do not react. There is only a weak reaction with organic sulphonic acid groups (e.g. benzene disulphonic acid gives 2% of the sulphite reaction). Sulpho-compounds do not affect the sulphite reaction. Carbonyl-sulphite complexes react in the same way as free sulphite. The assay is disturbed only by isothiocyanates occurring in mustard.

Other interfering substances are unknown. Sugars (up to 200 mg in the test) have no influence on the test. Organic acids such as citric, malic,

tartaric and lactic acids, occurring in food, also have no influence. Alcohols and sugar alcohols likewise have no effect. Interference is observed only by ascorbic acid. High concentrations occurring in beverages should therefore be removed by ascorbate oxidase.

DETERMINATION OF SULPHITES IN FOOD

The method has been developed for determining sulphites in food and the following concentrations have been measured:

	Amount ($mg\,SO_2/litre$)
White wine (total sulphite, free- and carbonyl-sulphite)	75–186
Red wine	60–142
Brandy	2–10
Beer	0–15
Grape juice	0–12
Citrus juices	8
Spices (e.g. pepper)	34
Preserves (strawberry, plum, cherry)	0–1
Potato snacks/chips	0–10

All samples with added sulphite showed recoveries of more than 96%.