Determination of Sulfite in Beer Based on Fluorescent Derivatives and Liquid Chromatographic Separation

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ABSTRACT

A method was developed for quantification of sulfite in beer based on derivatization with the maleimide-derived probe ThioGlo 1 followed by separation of fluorescent adducts by reversed-phase high-performance liquid chromatography and fluorescence detection. Sulfite gave two ThioGlo 1 derivatives and it was shown by mass spectrometry that both had identical mass spectra. Matrix effects were observed when constructing sulfite standard curves in different beers and, therefore, use of a matrix-matched calibration curve is proposed. ThioGlo 1 was found to generate fluorescent adducts with both bound and free sulfite, providing a quantification of the total sulfite content in beer. The limit of quantification of sulfite was 0.6 mg/L and the method can be used for quantification of sulfite in highly colored beers.

Keywords: Analysis, Beer, Matrix match, Sulfite, ThioGlo 1

RESUMEN

Se desarrolló un método para para la cuantificación de sulfito en cerveza sobre la base de derivatización con la sonda derivada de maleimida, ThioGlo 1, seguido por la separación de los aductos fluorescentes usando fase inversa cromatografía líquida de alto rendimiento y detección por fluorescencia. El sulfito dio dos derivados de ThioGlo 1 y se demostró por espectrometría de masas que ambos tenían espectros de masa idéntica. Efectos de matriz se observaron en la construcción de las curvas estándar de sulfito en diferentes cervezas, y por lo tanto el uso de una curva de calibración ajustada a la matriz se propone. ThioGlo 1 fue encontrado para generar aductos fluorescentes con tanto unido y libre de sulfito, proporcionando una cuantificación del total de sulfito contenido en la cerveza. El límite de cuantificación fue de 0.6 mg/L de sulfito y el método puede ser utilizado para la cuantificación de sulfito en cervezas muy coloreadas.

Palabras claves: Ajustada a la matriz, Análisis, Cerveza, Sulfito, ThioGlo 1

Sulfite (SO_3^{2-}), also often referred to as sulfur dioxide (SO_2), is an additive in foods, which functions as an antioxidant and as a preservative to reduce or prevent microbiological spoilage (27). In beer, sulfite is produced during the fermentation by the yeast as an intermediate in the amino acid synthesis, and it occurs naturally in concentrations of 0.5 to 10 ppm as the protonated species bisulfite (HSO_3^-) (11). The oxidative stability of beer has been linked to the presence of sulfite, and it has been suggested that sulfite inhibits oxidative reactions in beer by scavenging the reactive oxygen species hydrogen peroxide (4,17,25). Sulfite may also bind to compounds containing carbonyls and, thereby, render them nonperceptible to the overall beer staling (7,22). Thus, sulfite has two important functions in relation to beer stability: it works as both an antioxidant and a camouflage of off-flavors; for example, (E)-2-nonenal (7).

Sulfite is a well-known allergen and sensitive individuals who are exposed, particularly asthmatics, may suffer reactions ranging from dermatitis, urticaria, flushing, hypotension, abdominal pain, and diarrhea to life-threatening anaphylactic and asthmatic reactions (26). The Joint FAO/WHO Expert Committee on Food Additives (28) has specified an acceptable daily intake of up to 0.7 mg of sulfur dioxide per kilogram of body weight. Within the European Union, food and beverages containing sulfur dioxide and sulfites at more than 10 ppm must be labeled according to directive 2003/89/EC (24). Beer, including non- to low-alcoholic beer, may not contain sulfur dioxide at more than 20 ppm. However, sulfur dioxide at 50 ppm is permitted in beer with a second fermentation in the cask according to directive 95/2/EC (23).

The level of sulfites in beer can be quantified by numerous techniques, including titrimetry, electrochemistry, fluorometry, chemiluminescence spectrometry, colorimetry, gas chromatography, biosensors, and liquid chromatography, including ion chromatography and flow injection analysis, of which the latter two have been intensely explored in recent years (8,12,18,21,29). The most frequently used methods are those recommended by the American Society of Brewing Chemists (ASBC) and the European Brewery Convention (EBC). The Institute of Brewing (IOB) Analysis Committee has evaluated alternative approaches to the traditionally accepted Monier-Williams based method (6). In a comparative study including the Monier-Williams method, the IOB rapid method, the ASBC *p*-rosaniline method, and a method based on Ellman's reagent (5,5′-dithiobis-(2-nitrobenzoic acid) [DTNB]), it was concluded that determination by means of *p*-rosaniline provided the better repeatability. Today, ASBC recommends the *p*-rosaniline method (5). EBC recommends the distillation method (Monier-Williams method) and the EBC enzymatic method for determining the total sulfur dioxide in beer (10). The Monier-William method is time consuming and labor intensive. Both the Monier-William and *p*-rosaniline methods use hazardous chemicals.

Lund and Andersen (19) have recently quantified thiol-containing compounds in beer using fluorescence detection after derivatization with 10-(2,5-dihydro-2,5-dioxo-1H-pyrrol-1-yl)-9-methoxy-3-oxo-3H-naphthol[2,1-b]pyran-2-carboxylic acid methyl ester (ThioGlo 1). Sulfite was found to interfere because it also forms fluorescent adducts with ThioGlo 1. ThioGlo 1 is a maleimide derivative which has high affinity for thiol groups. The probe is weakly fluorescent when un-reacted, but highly fluorescent as sulfite- or thiol-derived adducts (Fig. 1) (9). A related maleimide derivative, N-(9-acridinyl)maleimide (NAM), has been reported to form derivatives with sulfite and, combined with reversed-phase high-performance liquid chromatography (RP-HPLC), it has been used for determination of sulfite in alcoholic beverages and environmental samples (1–3,20). A drawback of the method was the formation of three NAM derivatives after

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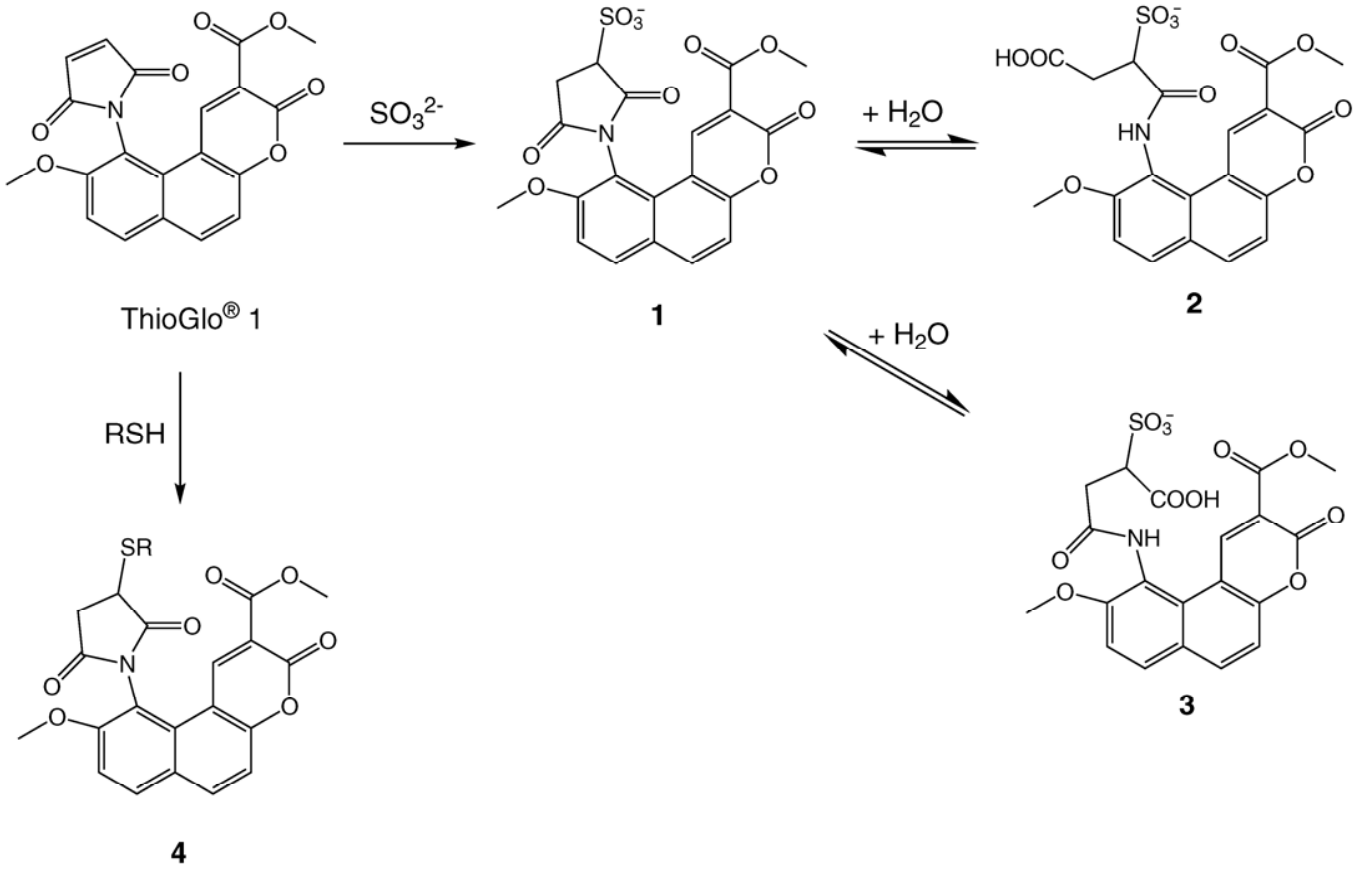

Fig. 1. Derivatization of sulfite and thiols with ThioGlo 1. Sulfite adds to the maleimide group by a Michael addition mechanism. The succinimide ring of the initial adduct 1 may undergo reversible hydrolysis to two isomeric compounds, 2 and 3. Thiols give fluorescent adducts 4.

reaction with sulfite, one initial adduct, and two isomeric hydrolyzed adducts. The method had to be performed at elevated pH (pH = 10) in order to accelerate the formation of the two hydrolyzed compounds, which were stable for more than 48 hr.

Here, we report the development of a method based on derivatization of sulfite in beer with ThioGlo 1 combined with RP-HPLC separation with fluorescence detection for determination of total sulfite content in beer. The use of RP-HPLC separation and fluorescence detection allows determination of sulfite in low concentrations, as well as in very dark beer types.

EXPERIMENTAL

Reagents and Chemicals

Stock solution of ThioGlo 1 (2.6 m*M*; Covalent Associates Inc., Woburn, MA) was prepared by dissolving 5.00 mg of ThioGlo 1 in 5.07 mL of anhydrous acetonitrile (Sigma-Aldrich, St. Louis) and storing it at 4°C, protected from light. A buffer solution (Tris-buffer) was made with 0.25 M tris(hydroxymethyl)aminomethane (Merck, Darmstadt, Germany) and the pH was adjusted to 7.5 with hydrochloric acid (HCl, 37%; Sigma-Aldrich, Steinheim, Germany). ThioGlo 1 solution was diluted 1:100 (v/v) with Tris-buffer to a concentration of 26 µ*M* prior to use. Sodium sulfite (J. T. Baker, Deventer, Holland) was used for making calibration curves and for synthesis of acetaldehyde-sulfite adduct using acetaldehyde (>99.9%; Sigma-Aldrich, St. Louis). 1-Octanol (>99.5%; Fluka, Buchs, Switzerland) was used as foam controller. Milli-Q water from a Q-plus purification system was used for standard solutions and mobile phases (Millipore Corp., Bedford, MA). Methanol (>99.9%; Merck) was of analysis grade and acetonitrile (>99.9%; Sigma-Aldrich, St. Louis) was of HPLC-gradient grade. Formic acid (>98%; Fluka), ammonium formate (>99.0%; Fluka), acetic acid (100%; Merck), and ammonium acetate (>98.0%; Merck) were of puriss grade. Trifluoroacetic acid (TFA) (>99.8%; Merck) was of spectroscopy grade. Triethanolamine (TEA) (>99.5%; Sigma-Aldrich, St. Louis) used in sample preparation was of analysis grade. For LC-MS mobile phases, water was glass distilled, methanol (>99.9%; Fisher Scientific, Loughborough, UK) was of LC-MS grade, and TFA (>99.5%; Fluka, St. Louis) was of protein sequence analysis grade.

Beers were bought from local shops. Beer A was a Danish lager made with barley and barley malt (can, 4.4% alcohol), beer B was a typical Danish all-malt lager beer (can, 4.6% alcohol), beer C was an all-malt bock-type of lager beer (bottle, 7.2% alcohol), beer D was a dark all-malt lager (can, 4.4% alcohol), beer E was a top-fermented all-malt ale (bottle, 5.5% alcohol), and beer F an all-malt porter (bottle, 7.8% alcohol).

Sample Derivatization

Beer samples were degassed by adding 10 µL of 1-octanol to 100 mL of beer and stirring for 5 min. Aliquots of 100 µL of degassed beer were diluted to 1 mL in Tris-buffer and 20 µL was transferred to a vial. Sulfite was added as a 1 mg/L solution in Tris-buffer and, finally, the total volume was adjusted to 100 µL with Tris-buffer. The added sulfite concentration ranged from 10 to 50 µg/L. Subsequently, 100 µL of ThioGlo 1 (26 µ*M* in 0.25 M Tris-buffer, pH 7.5) was added to the vial. The reaction was quenched after 5 min by addition of 10 µL of 12 *M* HCl. The final dilution factor of beer was 1:105 (v/v) and the concentration of

ThioGlo 1 was approximately 13 μ*M*. This corresponded to a sulfite standard addition calibration curve prepared in the range of SO_2 at 0 to 25 mg/L.

Free and Bound Sulfite: Addition of Acetaldehyde or Acetaldehyde-Sulfite Adduct

Acetaldehyde-sulfite adduct (sodium 1-hydroxyethanesulfonate) was synthesized according to the procedure described by Andersen et al (4). A beer of the same type as beer C was degassed as described above and divided into two portions. One portion was used as control and to the other portion was added either acetaldehyde (25 m*M*) followed by incubation for 30 min at room temperature or acetaldehyde-sulfite adduct (3 mg/L). Sulfite content was determined using the standard addition calibration procedure described above.

Instrumentation and Chromatographic Conditions

An Agilent 1100 Series liquid chromatographic system consisting of a model G1312A binary pump, a G1379A vacuum degasser, a G1313A autosampler, a G1321A fluorescence detector, and Agilent ChemStation data handling program (Agilent Technologies Inc., Palo Alto, CA) was used. Separation was performed on a Jupiter C18 (150 by 2.0 mm, 5-μm particle size, 300-Å pore size) column (Phenomenex, Torrance, CA). Water (mobile phase A) and methanol (mobile phase B) were both acidified with equal amounts of TFA (pH 2.0, 10 m*M*). The gradient was held at 25% B for 8 min (isocratic), instantly increased to 95% B and kept at 95% B for 6 min. The mobile-phase conditions were then returned to starting conditions and reequilibrated for 7 min, resulting in a total run time of 21 min. Injection volume was 20 μL. Flow rate was 0.5 mL/min, and detection was performed at excitation of 242 nm and emission of 492 nm. The sum of the areas of the two peaks corresponding to ThioGlo 1-derivatized sulfite was used for the quantification of sulfite.

Mass Spectrometry

The ThioGlo 1-sulfite adducts were characterized using an Acquity ultra-performance liquid chromatograph (Waters, Milford, MA) equipped with a photodiode array detector and a fluorescence detector, coupled to an Ultima Global quadrupole/orthogonal acceleration time-of-flight (TOF) mass spectrometer with electrospray ionization operated in negative ion mode, and MassLynx v4.1 was used for data acquisition and processing (Waters Micromass, Manchester, UK). The ThioGlo 1 derivatized compounds were separated on the Jupiter C18 column described above. Samples were prepared as previously stated with the following exceptions: TEA buffer (pH 7.5, 0.25 *M*) was used instead of Tris-buffer (pH 7.5, 0.25 *M*) and TFA was used instead of HCl to quench the ThioGlo 1 reaction. The gradient was held at 25% B for 12 min (isocratic), instantly increased to 95% B, and kept at 95% B for 5 min. Flow rate was decreased to 0.4 mL/min to increase performance of the mass spectrometer source. The mass spectrometer was operated at ion source temperature 120°C, desolvation gas temperature 420°C, cone gas flow 50 L/hr, desolvation gas flow 700 L/hr, capillary voltage 0.9 kV, cone voltage 20 V, scan time 1 sec, and interscan delay 0.1 sec. The mass spectrometer was operated in TOF scan mode (*m/z* 100 to 900). External calibration (*m/z* 180 to 793, 10-point calibration, fifth order of polynomial fit) was performed before analysis with 1 m*M* sodium hydroxide and 0.01% formic acid in methanol.

Color Determination

The absorbance of the different beers was measured at 430 nm according to EBC method 9.6 (10) using an HP-8453 spectrophotometer (Hewlett Packard, Portland, OR). Quantification of the beer color (EBC units), *C*, was then determined according to the equation $C = 25 \times f \times A_{430}$, where A_{430} is the absorbance at 430 nm and *f* is the dilution factor of the beer.

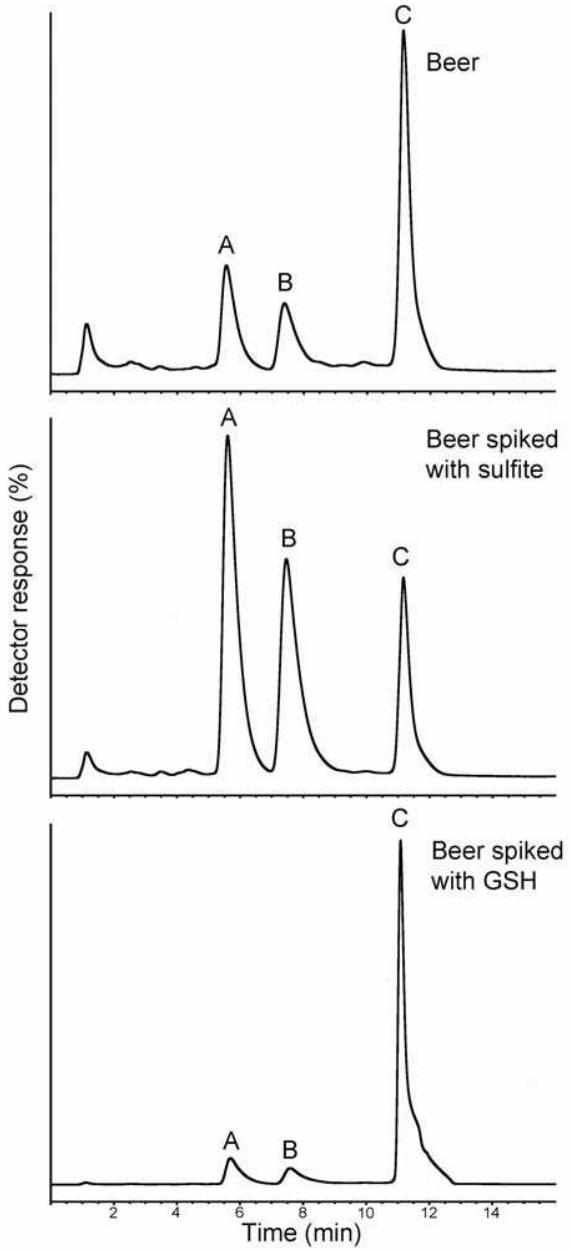

Fig. 2. Chromatogram of beer C analysis after method optimization. Peaks corresponding to derivatized sulfite (peaks A and B) and thiol-containing compounds (peak C) were identified through identification by spiking beer C with either sulfite or glutathione (GSH). Peaks are presented as percentage of the highest peak in the chromatogram.

RESULTS AND DISCUSSION

HPLC Separation and Detection of ThioGlo 1 Adducts

The HPLC separation of ThioGlo 1 adducts with sulfite and beer thiols on a C18 column was tested with various mixtures of water, methanol, and acetonitrile as eluents; eluent gradient profiles; and pH values of eluents between 2.0 and 5.0 either by adjusting with TFA or buffering with formic acid and ammonium formate or acetic acid and ammonium acetate. Optimization resulted in an eluent gradient based on only water and methanol

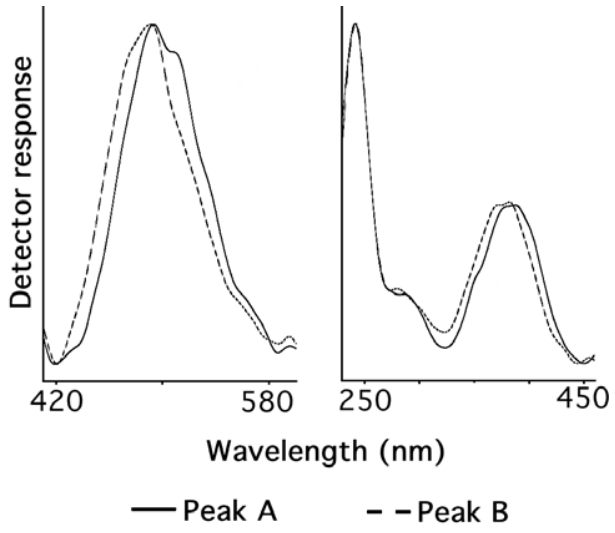

Fig. 3. Wavelength spectra at the peak apex of sulfite adducts (peaks A and B eluted first and second, respectively) in a reversed-phase HPLC chromatogram of a beer sample as shown in Figure 2. Emission spectra (left) were obtained with 384 nm excitation and excitation spectra (right) were obtained with 492 nm emission detection. The chromatographic separation was performed with a 25% methanol eluent for 8 min and then increased to 95% methanol for 6 min. The mobile phases were adjusted to pH 2.0 with trifluoroacetic acid. Flow rate was 0.5 mL/min.

with pH 2.0 adjusted with TFA, held at 25% B for 8 min (isocratic), instantly increased to 95% B, and kept at 95% B for 6 min. The mobile-phase conditions were then returned to starting conditions and the system reequilibrated for 7 min, resulting in a total run time of 21 min. These chromatographic conditions gave chromatograms where sulfite derivatives were baseline separated and the instant change of eluent composition after elution of the sulfite derivatives resulted in all the thiol-containing derivatives eluting within one single peak (Fig. 2, peak C). Sulfite derivatives were identified by injecting a derivatized standard of sulfite in buffer and always gave two peaks (Fig. 2, peaks A and B). Generally, eluents with pH higher than 2.0 or containing acetonitrile resulted in some thiol-containing derivatives eluting earlier than both the bulk thiol and sulfite derivatives.

Optimization of the fluorescence detection using ThioGlo 1-derivatized beer samples showed that optimal excitation wavelengths were 242 and 385 nm and that, in both cases, maximal emission occurred at 492 nm (Fig. 3). However, the excitation at 242 nm gave the highest emission response, and it was chosen for the further quantitative studies.

ThioGlo 1-sulfite Derivatives

Meguro et al (20) investigated the impact of pH and temperature on the derivatization of sulfite with the analogous maleimide compound NAM and found that NAM, over time, formed three products with sulfite, and that both the formation rate and relative amounts of the products were pH dependent. ThioGlo 1-derivatized sulfite gave rise to two peaks in the chromatograms, and the relative intensities of the two peaks were constant, provided the same buffer and pH were used during derivatization. The two sulfite derivatives were assessed with LC-MS in order to confirm that the peaks were derived from sulfite. The MS spectra of the two derivatized sulfite peaks were identical. With negative ion mode electrospray and full scan mode (*m/z* 100 to 900) with subtracted background, ions of *m/z* 460.0, 596.0, 612.0, 726.0, and 732.0 were found in the MS spectra of both peaks of derivatized

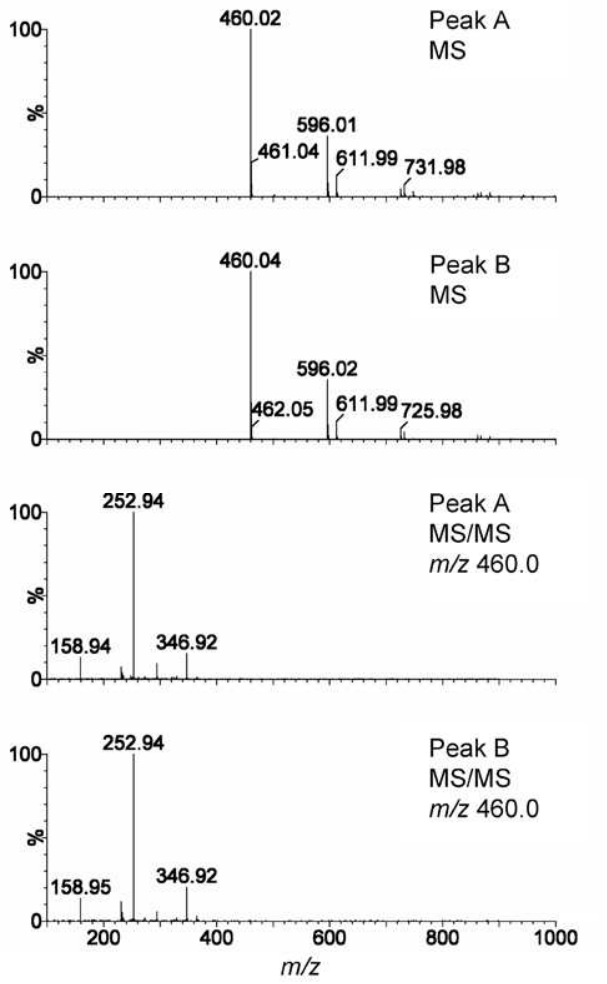

Fig. 4. Mass spectra of peaks (Fig. 2, peaks A and B) corresponding to derivatized sulfite in full scan between *m/z* 100 and 900 and daughter ions of *m/z* 460.0 (negative ion mode) of ThioGlo 1-sulfite derivatives in peaks A and B fragmented with collision energy of 30 V.

sulfite. The ion of *m/z* 460.0 was assigned to be a sulfite-ThioGlo 1 derivative, while the other ions were not identified but assumed to be adducts or cluster ions. A comparison of the theoretical isotope pattern of the ThioGlo 1-sulfite derivative with the elemental composition $C_{20}H_{14}NO_{10}S$ and the detected isotope pattern around *m/z* 460.0 showed indistinguishable compositions.

The two ThioGlo 1-sulfite derivatives (*m/z* 460.0) were further studied by fragmentation in MS/MS mode at collision energies of 10 and 30 V. In a scan mode of the daughter ions, the mother ion was intact at 10 V and only minor fragmentation was observed (*data not shown*). Only an ion of *m/z* 346.9 was observed in both spectra. At a collision energy of 30 V, the mother ion was not present. Fragments of *m/z* 346.9, 294.1, 252.9, and 158.9 were found in both spectra and with similar relative abundances (Fig. 4). The similar fragmentation patterns indicate that the ThioGlo 1 derivatives in the two HPLC peaks are converted to the same ion during the MS-analysis. Akasaka et al (2) suggested, based on IR and NMR data, that the succinimide ring structure of NAM-sulfite adducts could be hydrolyzed to two isomeric compounds, and a similar behavior has been reported for thiol-maleimide adducts (15). The HPLC separation of the ThioGlo 1-sulfite adducts into two peaks can be explained by a similar mechanism where two isomeric hydrolyzed adducts, 2 and 3, are formed (Fig. 1). However, the identical MS results for the two peaks and the lack of

TABLE I
Characteristics of the Six Standard Curves Made by Addition of Sulfite to Beer and the Beer Color (95% Confidence Interval)

	Beer					
	A	B	C	D	E	F
Response Factor	540.6 ± 28.0	527.3 ± 11.3	443.9 ± 32.1	449.6 ± 27.9	414.1 ± 12.6	407.2 ± 19.0
R^2	0.9986	0.9998	0.9973	0.9980	0.9995	0.9989
EBC Color[a]	8.62 ± 0.04	7.84 ± 0.05	13.8 ± 0.1	30.0 ± 0.4	128.5 ± 0.5	236.9 ± 0.5

[a] n = five replicates.

TABLE II
Quantitative and Statistical Results Derived from Analysis of Sulfite in Different Types of Beer

	Analyzed Beer, SO_2 Concentration (mg/L)						
Beer for Standard[a]	A	B	C	D	E	F	RMSE[b]
A	1.89[c]	5.32	6.81	3.42	0.46	1.86	1.01
B	1.57	5.44[c]	6.98	3.51	0.47	1.90	0.93
C	1.86	6.48	8.73[c]	4.17	0.56	2.26	0.49
D	1.84	6.40	8.19	4.51[c]	0.55	2.23	0.50
E	1.99	6.95	8.89	4.47	0.57[c]	2.42	0.68
F	2.03	7.07	9.04	4.54	0.61	2.32[c]	0.74
SD	0.16	0.71	0.97	0.52	0.05	0.24	…
Mean	1.83	6.12	7.92	4.01	0.52	2.14	…
RSD (%)	9	12	12	13	10	11	…

[a] Beer used for constructing standard curve. SD = standard deviation and RSD = relative standard deviation.
[b] Root mean squared error.
[c] Value attained from the standard addition method.

real mother ion of *m/z* 478.0 suggest that the hydrolysis is reversible, and that the conditions during the electrospray result in loss of water and a closure of the succinimide ring, regenerating the initial sulfite adduct. The MS results together with the HPLC spiking experiments prove that the two ThioGlo 1 adducts detected after the HPLC separation are derived from sulfite and, therefore, that they can be used for the direct quantification of sulfite.

Matrix-Matched Calibration Curve

Quantification of sulfite by means of a standard calibration curve made with water proved to be unsatisfactory due to the significantly different ($P < 0.01$) response factors of 101.4 ± 1.6 and 89.7 ± 2.6 (95% confidence interval) for calibration curves prepared in water and in beer C, respectively. The matrix effects of beer imply that correct measurements may be obtained through matrix-matched calibration or standard addition but not from aqueous calibration solutions. In order to overcome the matrix-related effects in beer, an attempt was made to establish a matrix-matched calibration curve. Different beers ranging from light to very dark types were tested as matrix match in order to determine whether the observed matrix effect was independent of beer type. Samples and spiked samples were prepared in triplicates and each injected twice for both types of calibration curves. The response factor appeared to be related to the darkness of the beer (Table I). A higher response factor was observed for the two brightest beers (A and B) while the darkest beer (F) gave the lowest response factor. These effects are likely due to inner-filter effects, where the intensities of either the light used for excitation or the light emitted by the fluorescent derivatives are affected by co-eluting nonfluorescent beer components.

To determine which beer was most suitable as a matrix match, the root mean squared error (RMSE) of the fit between matrix-match calibration curve results and results obtained by standard addition was calculated. The matrix-matched calibration curves were prepared in each of the six beers, keeping in mind the initial levels of sulfite. The sulfite level in each beer was then determined by each matrix-matched calibration curve. Levels below the calibration range were quantified by extrapolating the calibration curve. Using external standard calibration curves by extrapolating below the calibration range is not good practice. However, for a matrix-matched calibration curve, where the matrix match is not a true blank (such as beer, which has a background level of sulfite), extrapolations below the calibration range are often necessary. The value attained from the standard addition method was considered as the "true"' value. Beers C and D had the best results, with RMSE values of 0.49 and 0.50, respectively (Table II). Beer E had the lowest initial level of sulfite (SO_2 at 0.6 mg/L, determined by the standard addition method), as well as acceptable RMSE. Consequently, beer E was chosen as matrix match. The standard addition experiment is always assumed to yield a more accurate measure of concentration and, indeed, the external standard calibration curve approach—with or without matrix match—should be considered an operational alternative, because it is less labor intensive and can be performed on smaller sample sizes but with lower accuracy as trade-off.

Method Validation

The limit of detection (LOD) and limit of quantification (LOQ) were determined by standard addition method using beer E, which contained the lowest level of sulfite. Determination of LOD and LOQ was done according to the International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) 6.3 and 7.3 based on the standard deviation of the response (n = 9) and slope (13). The standard deviation of the response was based on the manually integrated analyte peaks in the nine replicate injections of beer E. Beer without added standard was injected nine times (injection volume = 20 µL). The LOD of SO_2 was determined to be 0.2 mg/L and the LOQ was 0.6 mg /L in beer.

The precision was based on sulfite determination of beers A and F utilizing the matrix-matched calibration curve of beer E (Table III). Three replicates of each sample were analyzed on three different days. The repeatability and intermediate precision were calculated according to ISO 5725-2 (13). Sulfite determination with the ThioGlo 1 method demonstrated good repeatability com-

TABLE III
Repeatability and Reproducibility of the Matrix-Matched ThioGlo 1 Method Compared with the Monier-Williams Method, the *p*-Rosaniline Method, the Institute of Brewing (IOB) Rapid Method, and the 5,5′-Dithiobis-(2-Nitrobenzoic Acid) (DTNB) Method[a]

	Mean	Repeatability		Reproducibility	
Sample	**SO_2 (mg/L)**	**SD_r**	**RSD_r (%)**	**SD_R**	**RSD_R (%)**
ThioGlo 1 method[b]					
Beer A	2.14	0.034	1.6	0.034	1.6[c]
Beer F	3.41	0.124	3.6	0.124	3.6[c]
Monier-Williams method[d]					
Level 1	1.84	0.231	12.6	0.810	44.0
Level 2	7.13	1.341	18.8	1.506	21.1
p-Rosaniline method[d]					
Level 1	1.37	0.206	15.0	0.371	27.1
Level 2	6.38	0.503	7.9	0.656	10.3
IOB Rapid method[d]					
Level 1	1.87	0.414	22.1	0.618	33.0
Level 2	6.01	0.749	12.5	1.653	27.5
DTNB method[d]					
Level 1	2.12	0.278	13.1	3.259	153.7
Level 2	8.16	1.197	14.7	6.87	84.2
Enzymatic method[e]					
Unknown sample	4	0.2	5	0.7	17

[a] Different batches of beers A and F were used than for previous experiment, thus the deviation in sulfite content compared with the results in Table 2. SD_r = Standard deviation of repeatability, RSD_r = relative standard deviation of repeatability, SD_R = standard deviation of robustness, and RSD_R = relative standard deviation of robustness.
[b] Each sample prepared in triplets at each day and reproducibility measured over three days, although at identical conditions.
[c] Intermediate precision. Between-days variance was insignificant.
[d] Assessed by the IOB analysis committee, conducted according to the ISO 5725 (14).
[e] Reported by the European Brewery Convention (10).

TABLE IV
Determined Recoveries Based on the Matrix-Matched Calibration Curves[a]

	Analyzed beer, Recoveries (%)					
Beer for Standard[b]	**A**	**B**	**C**	**D**	**E**	**F**
A	104	97	88	87	76	73
B	107	100	90	89	78	75
C	127	118	107	106	93	89
D	125	117	105	105	91	88
E	136	127	114	114	99	96
F	138	128	116	115	101	97

[a] Background sulfite levels were quantified using the standard addition method and the mean values of recoveries were based on SO_2 spike levels of 5, 10, 15, 20, and 25 mg/L.
[B] Beer used for constructing standard curve.

pared with already accepted methods (6,10), with values of 1.6 and 3.6% expressed as RSD for beers A and F, respectively. A variance components analysis using nested analysis of variance showed that the variance component between days was not significant compared with the within-day variance. Thus, the variation between days could simply be accounted for by the random error. Therefore, basic estimations of intermediate precision provided 1.6 and 3.6% RSD by analyzing beers A and F, respectively. The precision of the presented method appeared to be comparable with and possibly better than the recommended methods, including the Monier-Williams, *p*-rosaniline, IOB rapid, DTNB, and enzymatic method. However, it must be stressed that precision estimations for these methods were evaluated under other conditions and in a much larger scale, covering 8 (performed by IOB) or 12 (performed by EBC) different laboratories.

Recoveries were calculated using five different levels of spiking ranging from 5 to 25 mg/L of added sulfite (Table IV). For all of the beers and concentrations tested, several matrix matches with good (90 to 110%) to acceptable (80 to 90 or 110 to 120%) accuracy could be found. The only exception was quantification of sulfite in beer C at low levels. The quantification of sulfite in beers A and B was not accurate when using beers C, D, E, or F as matrix match (i.e., recoveries were lower than 90% or higher than 110%). To the same end, quantifications of sulfite in beers C, D, E, and F were acceptably accurate using beers C, D, E, or F as matrix match. Best results are obtained if a beer of corresponding color is used as matrix match. Not surprisingly, lower sulfite levels were determined with slightly lower accuracy. Because beers A and B were bright beers and beers C, D, E, and F dark beers, the general recommendation is to use light beer as matrix match for quantification of sulfite in light beer and vice versa.

Quantification of Free and Bound Sulfite

The current method is based on standard calibration curves prepared with addition of free sulfite to beer. However, sulfite in beer appears both as free and bound sulfite, where acetaldehyde-sulfite adducts, according to Kaneda et al (17), is the dominating bound form. The ability of ThioGlo 1 to react with bound sulfite was tested by adding acetaldehyde to beer C and, subsequently, quantifying sulfite by the standard addition calibration procedure. Acetaldehyde binds sulfite reversibly as adducts:

$$CH_3CHO + HSO_3^- \leftrightarrows CH_3CH(OH)SO_3^- \quad (1)$$

Addition of acetaldehyde (25 m*M* final concentration) to beer and incubation for 20 min result in complete reaction between acetaldehyde and free sulfite (17). Based on this knowledge, acet-

aldehyde (25 m*M*) was added to a beer containing 5.2 ± 0.3 mg/L of SO_2 as determined by the ThioGlo 1 method. After the addition of acetaldehyde, the level of sulfite in the beer as determined by the ThioGlo 1 method was 5.5 ± 0.1 mg/L of SO_2, which demonstrates that the potential binding of sulfite by acetaldehyde does not affect the measured level of sulfite. This finding was further supported by an experiment where pure crystalline acetaldehyde-sulfite adduct was synthesized and added to a beer similar to beer C. The beer originally contained 4.4 ± 0.2 mg/L of SO_2. After addition of acetaldehyde-sulfite adduct equivalent to 3.0 mg/L of SO_2, the level of sulfite was determined to be 7.4 ± 0.2 mg/L of SO_2, in very good agreement with the value expected. These results confirm that the ThioGlo 1-based method detects both bound and free sulfite and, thereby, quantifies the total level of sulfite in beer.

CONCLUSIONS

Sulfite in different types of beer can be determined by initial derivatization with ThioGlo 1 and subsequent RP-HPLC separation of the fluorescent adducts. The method determines total sulfite in beer and is based on using standard addition for the quantification. A matrix-matched calibration curve was found suitable for the simultaneous analysis of beer of different brands and types.

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