S-Sulfonate Determination and Formation in Meat Products

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A treatment with cyanide for the analysis of *S*-sulfonates in meat and meat derivatives, after a study of the effectiveness of this agent and that of dithiothreitol (DTT), is proposed. Once the protein-bound sulfite has been released, it is determined by HPLC ion exclusion with electrochemical detection. In the assay on the reproducibility of the method, standard deviations were 7.4, 9.2, and 11.4 for mean *S*-sulfonate values of 69, 107, and 130 μg of SO_2/g, respectively. Mean recovery was 91.2% for different amounts (56, 111, and 223 μg of SO_2/g) of *S*-sulfocysteine added. A study was made of the formation of *S*-sulfonates in model systems and in meat from different species—chicken and beef—with different fat contents. In the assays with meat, two different levels of sulfite addition were used: 600 and 1200 μg of SO_2/g. From the assays carried out in model systems with sulfite and cystine it may be concluded that one factor limiting the interaction is the accessibility to disulfide groups. The proportion of *S*-sulfonates in sulfited meat remains relatively constant and does not seem to be governed by the meat component, the level of sulfite addition, or the fat content. However, the latter two factors are inversely correlated with the retention of sulfite in the foods analyzed.

Keywords: *S-Sulfonates; sulfites; meat; cyanide; dithiothreitol (DTT)*

INTRODUCTION

After the addition of sulfites to foods, these compounds may be found as sulfurous acid, free inorganic sulfites, and a large variety of bound sulfite forms. Regarding the latter, a distinction should be made between the bound forms that are readily dissociated and the so-called irreversibly bound forms, which are very stable. *S*-Sulfonates ($R-S-SO_3^-$), which are formed by the reaction between sulfites and the disulfide bonds of cystine, peptides, and proteins, are considered to be irreversibly bound forms, and the sulfite of these compounds cannot be determined by the methods usually employed in the analysis of the additive (*1*−*5*).

In meat products, immediately after the addition of sulfites, irreversible losses of the additive occur due to the oxidation of sulfite and the formation of nondissociable bound forms. These losses have been calculated from the difference between the sulfite added and that determined, the latter not including the protein-bound fraction. Banks et al. (*6*) reported that in fresh sausages this initial reduction represents 26% of the amount added and that of the remaining level 23% is reversibly bound to the meat constituents. However, Wedzicha and Mountfort (*7*) found that the percentage of loss after addition to minced meat is 46%, although in other assays involving addition to different types of meat they determined very different values, ranging from 10 to 49% of the sulfite added. They attributed this variation in the reactivity of sulfur dioxide to the influence of the way in which the food was prepared and to its composition. To obtain reproducible results, the conditions of processing and sulfite addition must be established. In this sense, the mixing time, the presence of oxygen, and the degree of mincing of the meat are all factors directly correlated with the loss of the additive.

Food composition has important repercussions not only with regard to the reactivity of sulfur dioxide but also to the type of compounds generated. Regarding the first aspect, the fat content is the most important variable because the nonfat solids of meat samples from different animal species react in a quantitatively similar fashion, although its effect on the amount of *S*-sulfonates present has not been established.

In sulfited foods the portion of sulfite bound to proteins is almost unknown, and only the results obtained for biscuits and meats sulfited in the laboratory (*7*, *8*) and the contents determined in commercial shrimp at our own laboratory (*9*) are available. When minced meat was sulfited experimentally with amounts of 607 μg of SO_2/g, between 11 and 48% of the sulfite was found in the form of *S*-sulfonates in different species and cuts of meat, and in the particular case of some pork samples no *S*-sulfonates at all were detected. This percentage was calculated with respect to oxidized and irreversibly bound sulfite; if it were expressed with the total content of the additive taken into account, protein-bound sulfite would represent between 2.9 and 13.2%. It should be noted that a linear correlation may exist between the *S*-sulfonate content and that of cystine, and in the meats in which no sulfonates were detected, no cystine was detected either (*7*).

Sulfites are added to fresh minced meat and meat products because of their antibacterial activity, and current European directives authorize their addition in a reduced number of foods: burger meat with a minimum content of cereals and/or vegetables of 4%; breakfast sausages and two types of traditional Spanish cured sausage, at maximum residual doses of 450 mg of SO_2/kg (*10*, *11*). However, in other countries sulfur dioxide is prohibited in these kinds of foods (*12*). For maximum residual sulfite levels, the protein-bound form is not

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10.1021/jf0002847 CCC: $20.00 © 2001 American Chemical Society
Published on Web 12/09/2000

included, an aspect that should be taken into account because it could imply additional exposure to the additive (*13*–*15*).

Apart from the aspects related to the safety issues involved in the use of sulfites, it should also be noted that disulfide bonds play an important role in maintaining the structure of proteins and in determining some of their physicochemical properties and nutritive value. Moreover, sulfite increases the protein net charge and this may lead to an improvement of functional properties (*16*–*21*).

For the determination of protein-bound sulfite it is necessary to treat the sample with an agent—cyanide or dithiothreitol (DTT)—that will permit the sulfite to be released, after which it is determined by any of the methods used for the analysis of total sulfite. In the determination of *S*-sulfonates in shrimp (*9*), we observed that DTT did not permit the release of irreversibly bound sulfite, although other authors (*7*) reported that they had obtained good results using the reducing agent in meat samples. Accordingly, we were prompted to reassess this issue because the effectiveness of the treatment may be governed by the type of food in question.

The aim of the present work was to set up a method that would allow us to determine *S*-sulfonates in meat and meat derivatives. For this, it was necessary to establish the type of treatment that the meat samples should be subjected to in order to release the sulfite from *S*-sulfonates and determine it by ion exclusion HPLC with electrochemical detection (*22*). A further aim was to study the effect of different variables on the formation of *S*-sulfonates in meats because little information regarding this aspect is available. To do so, we conducted assays with solutions of sulfite and cystine and with meat to which cystine had been added and with meat from different species, with different fat contents and two different sulfite addition levels.

MATERIALS AND METHODS

Reagents. *S*-Sulfocysteine (cysteine-*S*-sulfonic acid) was purchased from Fluka Chemie AG (Buchs, Switzerland); dithiothreitol (DTT) and L-cystine were obtained from Sigma-Aldrich Chemical, Co. (St. Louis, MO); potassium cyanide, sodium hydroxide, sodium sulfite, tris(hydroxymethyl)aminomethane, and aluminum oxide 90 were supplied by Merck (Darmstadt, Germany).

Apparatus. A Milton Roy model CM-4000 HPLC system was equipped with an electrochemical detector (Metrohm, model 6565) with a glassy carbon electrode at 1150 mV and an Ag/AgCl reference electrode. Output from the detector was fed to a Milton Roy model CI-4000 integrator. Separation was accomplished on a 150×7.8 mm anion exclusion column (Fast Fruit Juice, Waters Chromatography, Milford, MA).

Samples. To set up a procedure designed to release protein-bound sulfite, unsulfited minced beef and commercial beefburgers were used. To compare the results in samples treated and not treated with DTT and then to determine sulfite with the optimized Monier–Williams method, we purchased 30 burgers elaborated at the same time by the same manufacturer. In the elaboration of these burgers, a commercial preparation containing sodium sulfite was used. The burgers were minced in a domestic blender and carefully mixed, and the homogenate was then divided into 50 g portions, which were frozen and stored at −30 °C until analysis. Each of these portions was considered to be one sample.

To study the reactivity of the sulfite the following were used:

(1) Solutions of sulfite and cystine. Cystine was dissolved in 0.1 N HCl, adjusted with 1 N NaOH to pH 6.5, and diluted with buffer at the same pH (0.010 M Na_2HPO_4 and 0.020 M KH_2PO_4). The final concentration of cystine was 4.7 μmol/mL. Sodium sulfite was added to this solution in such a way that the concentration of this compound would be 9.4 μmol of SO_2/mL (600 μg of SO_2/mL). The pH of the solution with the two reacting species was 7. After 1 h of reaction at 20 °C, the free sulfite and cysteine-bound sulfite were determined.

(2) Minced beef. Cystine and sulfite were added so that the final concentration of cystine would be 4.7 μmol/g of meat and that of sulfite would be 9.4 μmol/g of meat. The cystine was added to the meat, which was then homogenized, and later sulfite was added. After 1 h at 20 °C, the meat was divided into portions of 10 g, which were stored at −30 °C until analysis.

(3) Minced chicken and beef. Sodium sulfite was added in a sufficient amount to obtain concentrations of 600 and 1200 μg of SO_2/g. Once the mixture had been made up, it was kept at 20 °C for 1 h, after which time the samples were divided into representative portions, and these were stored at -30 °C.

(4) Minced beef. Fat was added so that the final content would be 27%, as compared to the 7% of the starting sample. The addition of fat was carried out at 40 °C in a water bath so that the mixture would be homogeneous. Following this, sulfite was added (600 μg of SO_2/g of meat), and the portions were kept as in the previous procedure.

Procedure for the Determination of *S*-Sulfonates in Meat and Meat Products. An aliquot of sample was subjected to treatment with cyanide or DTT to release the protein-bound sulfite. At the same time, another aliquot of the same sample was subjected to identical treatment with regard to pH and temperature but without cyanide or DTT. Then, SO_2 was determined in both aliquots by HPLC (*22*, *23*) or by the optimized Monier–Williams method (*24*). In the samples treated with DTT or cyanide, quantification was being made of the free sulfite plus the reversibly bound form and also that bound to proteins. In the other aliquot, only the total sulfite (free plus reversibly bound) was being determined. The protein-bound sulfite was calculated from the difference between the sulfite determined in both aliquots.

(a) Treatment with Cyanide. Three different conditions were used:

Treatment with cyanide I. Ten milliliters of cyanide solution at pH 12 (0.125 M KCN and 0.03 M NaOH) was added to 2 g of sample. This was homogenized in a Polytron blender, and 5 mL of 0.020 M Na_2HPO_4, adjusted to pH 12 with NaOH, containing 0.1% (v/v) of glycerol was added. The resulting suspension was incubated in a water bath with stirring at 37 ± 1 °C for 1 h. After this time, the mixture was cooled and kept at 4 °C for 10 min; 15 mL of 0.020 M Na_2HPO_4 (pH 12), was added, and the SO_2 was determined by HPLC (*23*). Another aliquot of the same sample was subjected to identical treatment, but the cyanide solution was replaced by Na_2HPO_4 adjusted to pH 12.

Treatment with cyanide II. In this case, the solution used was adjusted to pH 10 (0.125 M KCN and 0.1 N HCl), and 0.020 M Na_2HPO_4 adjusted to pH 10 with NaOH was likewise used. The times and temperatures of incubation were kept equal to those used in the previous treatment. After the mixture had been cooled, 15 mL of Na_2HPO_4 (pH 10) was added and the sulfite was determined by HPLC (*22*).

Treatment with cyanide III. Cyanide and Na_2HPO_4 at pH 10 were used, as in treatment II, but in this case the mixture was not incubated at 37 °C but instead at 20 °C for 30 min.

(b) Treatment with DTT. Five milliliters of 0.020 M DTT dissolved in 0.050 M Tris-HCl buffer (pH 9.2) containing 5 mM EDTA was added to 3 g of previously homogenized sample. The mixture was incubated at 37 ± 1 °C for 5 min. After the protein-bound sulfite had been released, determination was carried out by the optimized Monier–Williams method. As in the previous cases (treatment with cyanide), it was necessary to perform the determination of total sulfite in another aliquot of the same sample. This treatment is based on the one proposed by Nakamura and Tamura (*25*) and has been applied to sulfited meats by Wedzicha and Mountfort (*7*).

Table 1. Sulfite Determined in Beef to Which *S*-Sulfocysteine (111 μg of SO_2/g) Had Been Added

minced meat + *S*-sulfocysteine	treatment	SO_2, μg/g $\bar{x}$	SD	*n*
sample A	Na_2HPO_4, pH 12	61	5.3	4
sample A	KCN treatment I[a]	78	5.0	4
sample B	Na_2HPO_4, pH 12	57	2.6	5
sample B	KCN treatment I[a]	60	2.2	5
sample C	Na_2HPO_4, pH 10	6.2	1.3	5
sample C	KCN treatment II[a]	82	4.4	5

[a] Treatments KCN I and II correspond to the conditions described under Materials and Methods.

Optimized Monier—Williams Method. This was used only to confirm some results, because it is considered to be the reference method for the determination of total sulfite (*24*).

Statistics. An *F* test for comparing standard deviations and a *t* test for comparison of means were used (*26*).

RESULTS AND DISCUSSION

Effectiveness of Different Treatments for the Release of Sulfite from *S*-Sulfonates. The method set up at our laboratory for the determination of *S*-sulfonates in shrimp (*9*) was not suitable for the determination of these compounds in beefburgers and sulfited meats. To adapt it to these samples, we conducted a series of assays that would allow us to establish the most suitable pH and temperature conditions for the release of protein-bound sulfite.

To aliquots of 2 g of unsulfited minced meat we added *S*-sulfocysteine (111 μg of SO_2/g) dissolved in water and then mixed the material carefully. The protein-bound sulfite was determined after the samples had been subjected to treatments with cyanide I and II. The results are shown in Table 1. The SO_2 contents found in samples A and B show that treatment with phosphate at pH 12 and a temperature of 37 °C for 1 h elicits the release of protein-bound sulfite. This effect had not been observed either in shrimp or in standard solutions of *S*-sulfocysteine (*9*). Logically, the breakdown of *S*-sulfonate is favored in the presence of cyanide, although not all of the SO_2 is recovered. When the pH was decreased to 10 and the time and temperature of incubation were maintained, in sample C—to which no cyanide had been added—the sulfite determined corresponded to only 5.6% of that added, whereas in the meat treated with cyanide, 74% of the sulfite present in the *S*-sulfocysteine was recovered.

Finally, working with meat samples treated with cyanide at pH 10 and in parallel with samples not treated with cyanide kept at the same pH for 30 min at 20 °C, a good recovery of the *S*-sulfocysteine added was achieved, 97.8% as reflected later in the recovery assays of the proposed method (Table 5). Because of this, we adopted this procedure—treatment with cyanide III—for the dissociation of the protein-bound sulfite in meats and meat derivatives.

To assess the effectiveness of DTT, aliquots of 3 g each were taken from the minced sample destined for this assay and half of them were subjected to treatment with DTT. Table 2 shows the mean contents of sulfite determined together with the statistics calculated. According to these results, it is seen that the concentrations of sulfite determined by prior treatment with DTT do not differ significantly ($p = 0.05$) from those determined without using the reducing agent. Thus, under the conditions employed, DTT cannot be said to be effective in the release of protein-bound sulfite in burgers. However, the sulfite added to meat in the form of *S*-sulfocysteine can be recovered by treatment with DTT. Indeed, in experiments in which *S*-sulfocysteine was added to 3 g of minced beef in the amounts shown in Table 3, the mean calculated recovery of 12 determinations with the Monier—Williams method was 91.4%. This confirms the hypothesis that we had previously proposed (*9*) to the effect that the lack of effectiveness of DTT can be attributed to intrinsic factors of the food that govern the accessibility of the reducing agents to sulfonates. Similar effects have been reported in beans, milk, and soybean proteins when DTT is used for the reduction of disulfide bonds (*16*, *17*, *27*). As discussed previously by us (*9*), the $HgCl_2$ used by Wedzicha and Mountfort (*7*) could exert a destabilizing effect on the proteins, which would facilitate the exposure of *S*-sulfonates to the reducing agent, and this could account for the discrepancy between the results offered by those authors and the ones reported here.

Table 2. Sulfite Determined in Beefburgers, Treated and Untreated with DTT, by the Optimized Monier—Williams Method[a]

	SO_2 $\bar{x}$, μg/g	SD, μg/g	CV, %	*n*
beefburgers	370	13.5	3.7	13
beefburgers treated with DTT	371	9.1	2.5	13

[a] $F_{exp} = 2.201$; $t_{exp} = 0.213$.

Table 3. Recovery of Sulfite Added as *S*-Sulfocysteine to Beef Using the Optimized Monier—Williams Method, after Treatment with DTT

sulfite added[a] (μg/g)	sulfite found after addition[a] (μg/g)				mean recovery (%)
53	48	50	48	49	92.0
106	100	97	93	94	90.5
159	144	150	142	148	91.8

[a] Expressed in μg of SO_2/g of comminuted meat.

Method for the Determination of *S*-Sulfonates in Minced Meat and Burgers. Treatment with cyanide III was applied to different samples of burgers. The results obtained show that in this type of food it is possible to release the protein-bound sulfite with this treatment. Below we report on the assays carried out to establish the reproducibility and recovery of the method proposed in this work.

The reproducibility assay was performed in three different burger samples. To do so, 250 g of each of the samples was homogenized, taking the necessary portions from these homogenates for each determination. The mean values, standard deviations, and variation coefficients (CVs) are shown in Table 4. In part, the variability observed was due to the need to calculate the content in *S*-sulfonates from the differences between two aliquots of the same sample. These results show less variation than those found in the analysis of *S*-sulfonates in shrimp.

Sulfite recoveries were evaluated by adding different amounts of *S*-sulfocysteine to unsulfited minced meat. A suitable volume (250—500 μL) of an aqueous solution of *S*-sulfocysteine, at a concentration necessary for the levels of addition shown in Table 5 to be reached for the samples, was added to 2 g of the homogenate, and the material was then carefully mixed. After 5 min, the cyanide treatment was applied and sulfite was determined as explained under Materials and Methods. The

Table 4. Reproducibility of the Method for *S*-Sulfonate Analysis in Burgers

burger	parameter	total[a] SO_2	SO_2 from *S*-sulfonates
sample D	$\bar{x}$, μg/g	493	69
	SD, μg/g	22.3	7.4
	CV, %	4.5	10.7
	n	7	7
sample E	$\bar{x}$, μg/g	254	107
	SD, μg/g	13.9	9.2
	CV, %	5.5	8.6
	n	7	7
sample F	$\bar{x}$, μg/g	687	130
	SD, μg/g	27.4	11.4
	CV, %	4.0	8.8
	n	7	7

[a] Free + reversibly bound.

Table 5. Recovery of Sulfite Added as *S*-Sulfocysteine to Minced Meat, Determined with the Method Described for the Analysis of *S*-Sulfonates

sulfite added[a] (μg/g)	sulfite found after addition[a] (μg/g)					mean recovery (%)
56	46	49	52	56	49	90.0
111	113	107	105	111	107	97.8
223	201	192	192	190	181	85.7
						91.2

[a] Expressed in μg of SO_2/g minced meat.

mean recovery calculated for 15 determinations was 91.2%. Only *S*-sulfocysteine was used because this compound is the only sulfonate commercially available, although it would have been desirable to evaluate the behavior of other sulfonates under the same analytical conditions.

Other authors have established the effectiveness of dissociation using bovine serum albumin mixed with $Na_2S_2O_5$ (*7*), but with this method it is not possible to establish the effect that the food might exert on the accessibility of the reducing agent to the sulfonates. In light of the results reported above and the experience of other authors in the breakdown of disulfide bonds with DTT, this aspect should be evaluated. In food proteins it is not possible to achieve a complete breakdown of disulfide bonds using only DTT (*16*, *17*, *27*). Also, the effectiveness of this reagent in the determination of *S*-sulfonates in serum has been questioned (*28*).

Formation of *S*-Sulfonates. Most of the assays were conducted with a sulfite concentration of 600 or 1200 μg of SO_2/g. The lower amount was chosen on the basis of the legally admitted limit for total SO_2 contents in burgers and also the data reported by Banks et al. (*6*) concerning loss. Other authors have also adopted this criterion even though in initial assays they found that the losses of the additive were greater (*7*). The use of sulfites in amounts higher than those recommended is relatively common in daily practice (*12*), and it is hence of great interest to know their distribution and reactivity under these conditions.

Table 6 shows the results obtained with sulfite and cystine solutions prepared as explained under Samples. The molar concentration of cystine corresponds to half that of sulfite. Under these conditions, sulfitolysis is favored because at pH 7 the effect of the carboxyl groups is still not manifest and 50% of the sulfite is present in the form of SO_3^{2-}, which is the species that reacts immediately with the disulfide bond (*29*–*32*). From a theoretical point of view, the maximum amount of

Table 6. Formation of *S*-Sulfonates in Model Assays

	addition			SO_2		
	sulfite, μmol	cystine, mL or g	parameter	free	total[a]	SO_2 from *S*-sulfonates
solution	9.4	4.7	$\bar{x}$, μg/mL	397		132
			SD, μg/mL	26.1		17.4
			CV, %	6.6		13.2
			n	6		6
solution	9.4	4.7	$\bar{x}$, μg/mL	389		145
			SD, μg/mL	43.3		11.7
			CV, %	11.0		8.1
			n	6		6
meat	9.4	4.7	$\bar{x}$, μg/g	151	229	283
			SD, μg/g	4.0	24.0	22.8
			CV, %	2.7	10.5	8.1
			n	5	5	5
meat	9.4	4.7	$\bar{x}$, μg/g	163	233	313
			SD, μg/g	7.7	21.8	10.9
			CV, %	4.7	9.4	3.5
			n	5	5	5

[a] Free + reversibly bound.

sulfite that could bind to cystine would be 300 μg of SO_2/mL. The theoretical calculation was made considering that bisulfite does not react with the amino acid and that the possibility of oxidation of the initially formed cysteine is practically zero.

The mean contents found in two different assays were 132 ± 17.4 and 145 ± 11.7 μg of SO_2/mL, corresponding to 44 and 48%, respectively, of the theoretical values. These percentages of formation of *S*-sulfocysteine are of the same order as those calculated by other authors (*33*, *34*), with a molar ratio of species equal to that of our own assays, although in the other cases work was performed at alkaline pH and in the presence of Cu^{2+}. Under these latter conditions, yields would be expected to be greater. Nevertheless, because these works were published some time ago, using the techniques then available, we believe that they should not be interpreted on the basis of a strictly quantitative criterion.

If one considers distribution with respect to the sulfite determined (total plus *S*-sulfonates), free SO_2 represents percentages of 75 ± 2.9 and 65 ± 7.5, respectively, whereas the fraction bound to cysteine would correspond to 25 ± 3.2 and 24 ± 2.0, respectively, in each of the assays. There is a small amount of undetermined sulfite, which must have been oxidized to sulfate.

We performed another experiment with meat to which cystine and sulfite were added as explained under Samples. The amount of cystine added was lower than that contained in the meat because in various cuts of meat contents of 12.5, 29, and 35 μmol/g have been found (*7*). The free and total sulfite and *S*-sulfonates were determined in these samples. The results depicted in Table 6 correspond to two additions to minced beef steaks and are referred to grams of meat with a view to their comparison with those found in other experiments without added cystine. For this, they were corrected by taking into account that fact that the amino acid is added in solution.

Considering only the cystine added to the meat, conversion of this into the corresponding sulfonate, expressed as a percentage with respect to the theoretical values, was 94 ± 7.6 and 104 ± 3.6 in each assay. These percentages are double those found in solutions of sulfite and cystine at the same concentration.

Under the same conditions but without the addition

Table 7. Formation of *S*-Sulfonates in Meat (SO_2 Added: 600 μg/g)

meat	parameter	total[a] SO_2	*S*-sulfonates	total[a] SO_2 + *S*-sulfonates
beef	$\bar{x}$, μg/g	496	84	581
	SD, μg/g	21.7	16.4	26.3
	CV, %	4.3	19.5	4.5
	n	10	10	10
chicken	$\bar{x}$, μg/g	446	125	569
	SD, μg/g	9.3	12.4	18.7
	CV, %	2.1	10.0	3.3
	n	10	10	10
beef (7% fat)	$\bar{x}$, μg/g	504	84	588
	SD, μg/g	10.5	15.5	12.5
	CV, %	18.4	18.4	2.1
	n	9	9	9
beef (27% fat)	$\bar{x}$, μg/g	443	61	504
	SD, μg/g	14.8	12.6	21.0
	CV, %	3.3	20.7	4.2
	n	9	9	9

[a] Free + reversibly bound.

of the amino acid, the mean content of *S*-sulfonates was 84 ± 16.4 μg of SO_2/g (Table 7). Taking this value into account, the formation of *S*-sulfocysteine corresponding to the cystine added can be fixed at values of 66 and 76% with respect to the maximum theoretical value.

The results of this experiment indicate that the presence of meat would favor the sulfitolysis reaction. This hypothesis should be viewed with caution, however, because under the experimental conditions employed it is necessary to introduce variables that might also affect the reaction. Because cystine is not soluble in neutral aqueous media, it must be dissolved in acid medium and the solution must be neutralized before it is added to the sample. Accordingly, the process of incorporation of the amino acid involves an additional supply of water and Cl^- and Na^+ ions to the meat. The chloride ion has a solubilizing effect on proteins, and the ability of these to retain water is increased upon the addition of salts such that it could be speculated that these changes in the reaction mixture could perhaps favor the formation of *S*-sulfonates (*35*). It is possible that ionic linkages of the protein structure might be ruptured by salts, which would cause the protein to partially unfold, thus exposing the buried disulfide bonds to sulfite (*36*).

Bearing in mind that the addition of cystine considerably increased the formation of *S*-sulfocysteine and that the amino acid was added in amounts far below those contained in the meat, it does not seem that the overall amount of the amino acid could be a limiting factor in the formation of *S*-sulfonates; instead, this would be governed by the readily accessible cystine fraction.

In this same assay, with increasing contents of sulfonates, the equilibrium between the different sulfite fractions is also modified, total SO_2 (free plus reversibly bound) being much lower—229 ± 24 and 233 ± 21.8 μg of SO_2/g—than that found in sulfited meats without added cystine (496 ± 21.7 μg of SO_2/g), as shown in Table 7.

To study the *reactivity of sulfite after its addition to meats*, we conducted different assays under controlled conditions with regard to the degree of mincing, temperature, and reaction time. One of the factors that may affect the reactivity of the additive is the type of meat, so the assays were performed with beef steaks and chicken breast.

Table 7 shows the results corresponding to 10 additions of sulfite to beef and chicken, respectively. From the contents of total SO_2, it may be seen that the amounts of sulfite not determined by the usual analytical method represent 17 ± 3.5% in the case of beef and 26 ± 1.5% in the case of chicken. Although both values are fairly close to those foreseen, it could be surmised that owing to the lower proportion of fat in the chicken the diffusion of sulfite for the formation of irreversible compounds such as *S*-sulfonates would be facilitated. Comparison of the levels of *S*-sulfonates found in the two assays, despite the absence of large differences, reveals that they are higher in the chicken samples. If one considers the sum of total sulfite and *S*-sulfonates, nearly all of the sulfite added is seen to be recovered, regardless of the meat component used. The percentages of retention of the additive are 97 ± 4.4 for beef and 95 ± 3.1 for chicken. Accordingly, the losses attributable to oxidation are minimum and lack significance.

From the above, it may be deduced that the small differences between both types of experimentally sulfited samples lie in the capacity of *S*-sulfonate formation, involving 15 ± 2.5% in beef and 22 ± 1.6% in chicken of the sulfite determined (total SO_2 plus *S*-sulfonates).

With a view to determining whether the fat content of the meat might be one of the factors determining the reactivity of the sulfite, an assay was performed with minced beef samples containing different proportions of fat. The absolute amount of sodium sulfite added to the sample with fat was increased proportionally to the increase in weight to achieve a concentration of 600 μg of SO_2/g of meat. The contents in total sulfite and *S*-sulfonates determined in both samples are shown in Table 7.

It may be seen that in the samples without added fat the reactivity of the sulfite and the fraction of *S*-sulfonates are identical to those seen in the previous assay. However, in the presence of high amounts of fat the sulfite determined represents 84% of the amount added. This is consistent with the hypothesis proposed by Wedzicha and Mountfort (*7*) concerning the effect of fat in preventing transport of the additive and reducing the possibilities of interaction with the components of the meat. However, according to our own findings this variable would above all affect the levels of free sulfite or of reversibly bound forms because although the concentrations of *S*-sulfonates are slightly lower, they are not significantly modified. It is likely that the overall effect of the fat would be reflected in an increase in the oxidation of free forms and not significant in favoring a selective interaction with certain components of the meat. In any case, the percentage of fat cannot be used as a discriminant parameter of the reactivity of the sulfite, at least with regard to the formation of *S*-sulfonates. Accordingly, the above considerations concerning beef and chicken meat would not be valid because the differences in the fat content between them would not imply significant changes in the reactivity of the additive.

We therefore carried out several assays with beef and chicken to which sodium sulfite in solution had been added in sufficient amounts to ensure a final concentration of 1200 μg of SO_2/g. Three different samples were used for each type of meat.

Table 8 shows the contents of total sulfite and *S*-sulfonates determined in the three independent assays in beef and chicken, respectively. After incorpora-

Table 8. Formation of *S*-Sulfonates in Meat (SO_2 Added: 1200 μg/g)

meat	parameter	total[a] SO_2	*S*-sulfonates	total[a] SO_2 + *S*-sulfonates
beef	$\bar{x}$, μg/g	759	192	950
	SD, μg/g	14.5	18.2	25.5
	CV, %	1.9	9.5	2.7
	n	4	4	4
beef	$\bar{x}$, μg/g	770	170	940
	SD, μg/g	6.4	24.8	28.0
	CV, %	0.8	14.6	3.0
	n	4	4	4
beef	$\bar{x}$, μg/g	771	143	914
	SD, μg/g	11.9	16.9	18.1
	CV, %	1.5	11.8	2.0
	n	4	4	4
chicken	$\bar{x}$, μg/g	811	217	1028
	SD, μg/g	18.7	11.2	29.3
	CV, %	2.3	5.2	2.9
	n	4	4	4
chicken	$\bar{x}$, μg/g	787	216	1003
	SD, μg/g	15.9	22.1	37.5
	CV, %	2.0	10.2	3.7
	n	4	4	4
chicken	$\bar{x}$, μg/g	797	183	980
	SD, μg/g	18.8	22.3	33.9
	CV, %	2.4	12.2	3.5
	n	4	4	4

[a] Free + reversibly bound.

tion of the additive, the percentages of sulfite not determined when the total sulfite analysis method was implemented were 36 ± 0.7 in beef and 34 ± 1.0 in chicken. In both cases, these values are higher than those obtained when the level of addition of the preservative was only half. Additionally, the amounts recovered when the *S*-sulfonates were also taken into account are lower than those added, representing 79 ± 1.6% for beef and 84 ± 2.0% for chicken. This means that in the presence of high levels of the additive, oxidation processes are favored over other interactions with meat components, and hence the retention of sulfite would vary as a function of the level of addition. However, the proportion of *S*-sulfonates remains practically the same, and it is not possible to establish real differences between either species of meat. The values corresponding to beef and chicken are 18 ± 2.3 and 20 ± 2.6%, respectively.

The existence of a limited degree of *S*-sulfonate formation, even when sulfite levels are very high, supports the hypothesis proposed on the basis of model assays in relation to another of the reacting species, namely, cystine. The total contents of both compounds will not be limiting factors to the interaction; instead, the accessibility of the disulfide groups would be responsible for this, and this would also be governed by the effect of other experimental factors (the presence of other compounds, pH, and temperature).

ACKNOWLEDGMENT

Thanks are due to Nick Skinner for the translation of the paper.

LITERATURE CITED

(1) Daniels, D. H.; Warner, C. R.; Joe, F. L., Jr. Colorimetric determination of sulfite in vegetables with modified *p*-rosaniline procedure. Presented at the 192nd National Meeting of the American Chemical Society, Los Angeles, CA, 1986.
(2) Fazio, T.; Warner, C. R. A review of sulphites in foods: analytical methodology and reported findings. *Food Addit. Contam.* **1990**, *7*, 443−454.
(3) Simon, R. A. Adverse reactions to food and drug additives. *Immunol. Allergy Clin.* **1996**, *16*, 137−176.
(4) Taylor, S. L.; Higley, N. A.; Bush, R. K. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity and hypersensitivity. *Adv. Food Res.* **1986**, *30*, 1−75.
(5) Wedzicha, B. L. Uses in food. In *Chemistry of Sulphur Dioxide in Foods*; Elsevier Applied Science: London, U.K., 1984; pp 275−311.
(6) Banks, J. C.; Dalton, H. K.; Nychas, G. J.; Board, R. G. Sulfite an efective agent in the microbiological and chemical changes occurring in uncooked comminuted meat products. *J. Appl. Biochem.* **1985**, *7*, 161−179.
(7) Wedzicha, B. L.; Mountfort, K. A. Reactivity of sulphur dioxide in comminuted meat. *Food Chem.* **1991**, *39*, 281−297.
(8) Thewlis, B. H.; Wade, P. An investigation into the fate of sulphites added to hard sweet biscuit doughs. *J. Sci. Food Agric.* **1974**, *25*, 99−105.
(9) Armentia-Alvarez, A.; Peña-Egido, M. J.; García-Moreno, C. S-Sulfonate determination in shrimp. *J. Agric. Food Chem.* **1997**, *45*, 791−796.
(10) Parlamento Europeo y Consejo de la Unión Europea. Directiva 95/2/CE del Parlamento Europeo y del Consejo de 20 de Febrero de 1995 relativa a aditivos alimentarios distintos de los colorantes y edulcorantes. *DOCE L61* **1995**, March 18, 1−40.
(11) Parlamento Europeo y Consejo de la Unión Europea. Directiva 98/72/CE del Parlamento Europeo y del Consejo por la que se modifica la Directiva 95/2/CE relativa a aditivos alimentarios distintos de los colorantes y edulcorantes. *DOCE L295* **1998**, Nov 4, 18−30.
(12) Armentia-Alvarez, A.; Fernández-Casero, A.; García-Moreno, C.; Peña-Egido, M. J. Residual levels of free and total sulphite in fresh and cooked burgers. *Food Addit. Contam.* **1993**, *10*, 157−165.
(13) Gibson, W. B.; Strong, F. M. Accumulation of ingested sulphite- and sulphate-sulphur and utilization of sulphited proteins by rats. *Food Cosmet. Toxicol.* **1974**, *12*, 625−640.
(14) Gibson, W. B.; Strong, F. M. Metabolism and elimination of α-hydroxyethane sulphonate by rats. *Food Cosmet. Toxicol.* **1976**, *14*, 41−43.
(15) Gunnison, A. S.; Palmes, E. D. Persistence of plasma S-sulfonates following exposure of rabbits to sulfite and sulfur dioxide. *Toxicol. Appl. Pharmacol.* **1973**, *24*, 266−278.
(16) Carbonaro, M.; Marletta, L.; Carnovale, E. Factors affecting cystine reactivity in proteolytic digests of *Phaseolus vulgaris*. *J. Agric. Food Chem.* **1992**, *40*, 169−174.
(17) Carbonaro, M.; Cappelloni, M.; Sabbadini, S.; Carnovale, E. Disulfide reactivity and in vitro protein digestibility of different thermal-treated milk samples and whey proteins. *J. Agric. Food Chem.* **1997**, *45*, 95−100.
(18) Friedman, M. Improvement in the safety of foods by SH-containing amino acids and peptides. A review. *J. Agric. Food Chem.* **1994**, *42*, 3−20.
(19) González, J. M.; Damodaran, S. Recovery of proteins from raw sweet whey using a solid state sulfitolysis. *J. Food Sci.* **1990**, *55*, 1559−1563.
(20) Petrucelli, S.; Añon, M. C. Parcial reduction of soy protein isolate disulfide bonds. *J. Agric. Food Chem.* **1995**, *43*, 2001−2006.
(21) Waniska, R. D.; Yi, T.; Lu, J.; Xue-Ping, L.; Xu, W.; Lin, H. Effects of preheating temperature, moisture, and sodium metabisulfite content on quality of noodles prepared from maize flour or meal. *Food Sci. Technol. Int.* **1999**, *5*, 339−346.

(22) Paino-Campa, G.; Peña-Egido, M. J.; García-Moreno, C. Liquid chromatographic determination of free and total sulphites in fresh sausages. *J. Sci. Food Agric.* **1991**, *56*, 85−93.
(23) Armentia-Alvarez, A.; Peña-Egido, M. J.; García-Moreno, C. Improved method for determination of sulfites in shrimp. *J. Assoc. Off. Anal. Chem.* **1993b**, *76*, 565−569.
(24) Food labeling; declaration of sulfiting agents. *Fed. Regist.* **1986**, *51*, 25012−25020.
(25) Nakamura, H.; Tamura, Z. Spectrophotometric determination of Bunte salts. *Chem. Pharm. Bull.* **1974**, *22*, 2208−2210.
(26) Miller, J. C.; Miller, J. M. Basic statistical methods for analytical chemistry. Part I. Statistics of repeated measurements. A review. *Analyst* **1988**, *113*, 1351−1356.
(27) Wolf, W. J. Sulfhydryl content of glycinin: effect of reducing agents. *J. Agric. Food Chem.* **1993**, *41*, 168−176.
(28) Ji, A. J.; Savon, S. R.; Jacobsen, D. W. Determination of total serum sulfite by HPLC with fluorescence detection. *Clin. Chem.* **1995**, *41*, 897−903.
(29) Cecil, R.; McPhee, J. R. The estimation of thiols and disulphides by potentiometric titration with silver nitrate. *Biochem. J.* **1955**, *59*, 234−240.
(30) Cecil, R.; McPhee, J. R. A kinetic study of the reactions on some disulphides with sodium sulphite. *Biochem. J.* **1955**, *60*, 496−506.
(31) Cecil, R.; Wake, R. G. The reactions of inter- and intrachain disulphide bonds in proteins with sulphite. *Biochem. J.* **1962**, *82*, 401−406.
(32) McPhee, J. R. Further studies on the reactions of disulphides with sodium sulphite. *Biochem. J.* **1956**, *64*, 22−29.
(33) Clarke, H. T. The action of sulfite upon cystine. *J. Biol. Chem.* **1932**, *97*, 235−248.
(34) Segel, I. H.; Johnson, M. J. Syntesis and characterization of sodium cysteine-S-sulfate monohydrate. *Anal. Biochem.* **1963**, *15*, 330−337.
(35) Rust, R. E. Productos embutidos. In *Ciencia de la Carne y Productos Cárnicos*; Price, J. F., Scheigert, B. S., Eds.; Acribia: Zaragoza, Spain, 1994; pp 420−421.
(36) Link, T. M.; Park, J. W. Solubility of salmon myosin as affected by conformational changes at various ionic strenghs and pH. *J. Food Sci.* **1998**, *63*, 215−218.

Received for review March 2, 2000. Revised manuscript received September 25, 2000. Accepted October 2, 2000. We are grateful to the CICYT (Ref. ALI97-0768) for financial support.

JF0002847