



August 20, 2012

Attn: Dr. George Alexeeff, Director
Ms. Susan Luong
Office of Environmental Health Hazard Assessment
P.O. Box 4010, MS-19B
Sacramento, CA
95812-4010
Fax: (916) 323-8803
Street Address: 1001 I Street, Sacramento, CA 95814
susan.luong@oehha.ca.gov
P65Public.Comments@oehha.ca.gov

Re: SULFUR DIOXIDE MADL

Dear Dr. Alexeeff:

The Grocery Manufacturers Association (GMA) represents the world's leading food, beverage and consumer products companies. The Association promotes sound public policy, champions initiatives that increase productivity and growth and helps to protect the safety and security of consumer packaged goods through scientific excellence. The GMA Board of Directors is comprised of chief executive officers from the Association's member companies. The \$2.1 trillion consumer packaged goods industry employs 14 million workers and contributes over \$1 trillion in added value to the nation's economy.

GMA appreciates the opportunity to provide the following comments in response to OEHHA's proposed safe harbor level for sulfur dioxide.

I. Executive Summary

The subject Notice¹ announced the beginning of a 45-day public comment period on the proposed Maximum Allowable Dose Level (MADL) for sulfur dioxide. The Notice proposes a MADL of 220 micrograms per day based on a Lowest Observed Effect Level (LOEL) of 25 ppm of SO₂ based on a decrease in fetal body weight in the most sensitive developmental toxicity study. A statistical re-analysis by Exponent of the raw data from this study revealed that the decrease in fetal body weight at 25 ppm of SO₂ is not statistically significant. Therefore, 25 ppm of SO₂ is the No Observed Effect Level (NOEL), not the LOEL, for decreased fetal body weight in the most sensitive developmental toxicity study. The use of this NOEL results in an MADL of 2200 micrograms per day. Additionally, using the benchmark dose approach also gives an MADL of 2200 micrograms per day, if not greater. A MADL of 2200 micrograms per day, which is compliant with the Proposition 65 regulations and appropriately conservative, should be established based on the best scientifically appropriate methods.

These comments also provide a detailed review of food chemistry and SO₂. While there is great complexity and extensive reactivity known in the food chemistry of products that have been treated with sulfur dioxide gas or various sulfite food additives, the most important fact for purposes of Proposition 65 is that “molecular SO₂” does not exist as such in foods and beverages. The molecular species that exist in foods and beverages do vary, depending on the product’s pH, but can include sulfurous acid, hydrated sulfur dioxide, various sulfites, bisulfites and metabisulfites, as well as sulfite-related forms bound chemically to various food and beverage constituents. These facts are well supported in numerous published reviews and research studies by leading sulfite experts over the past three decades. During analytical procedures where food is either acidified or made basic beyond the normal pH range of food, sulfites and sulfite precursors are produced via the decomposition of “bound” sulfites. These are artifacts of the analytical method, and they do not demonstrate the presence of molecular SO₂ in foods as consumed. Therefore, the listed chemical, sulfur dioxide, does not exist in foods and beverages and is thus not subject to Proposition 65 requirements.

¹ Cal/EPA OEHHA Prop 65 NOTICE OF PROPOSED RULEMAKING – SULFUR DIOXIDE (SO₂), July 6, 2012 (http://oehha.ca.gov/prop65/law/pdf_zip/062812SO2_CRNR.pdf)

II. Proposed MADL for SO₂

Introduction and Summary

The Office of Environmental Health Hazard Assessment (OEHHA) has proposed a Maximum Allowable Dose Level (MADL) for sulfur dioxide (SO₂) of 220 micrograms/day in its Initial Statement of Reasons, Title 27, California Code of Regulations Proposed Amendment to Section 25805(b) dated June 28, 2012. The rationale for this proposed MADL is detailed in an appendix to the Proposed Amendment that is entitled “Appendix ‘A’ Derivation of the Proposed Maximum Allowable Dose Level for Sulfur Dioxide.” The Proposed Amendment and Appendix A will be referred to collectively in this document as “the Proposed MADL Document.”

The comments submitted herein identify minor modifications to OEHHA’s proposed calculations of the MADL that would result in a MADL for SO₂ of 2200 micrograms/day or greater. Each of these minor revisions to the proposed MADL would produce a MADL which is (1) compliant with the Proposition 65 regulations, (2) scientifically more appropriate than using default assumptions as proposed, and (3) appropriately conservative.

Specifically, rather than basing the MADL on an estimate of the No Observed Effect Level (NOEL) by applying a default 10-fold factor to a Lowest Observed Effect Level (LOEL), new analysis of data indicates the NOEL for fetal body weight is 25 ppm of SO₂ in the most sensitive developmental toxicity study. Using this NOEL and exactly the same assumptions and calculations used in the Proposed MADL Document (except for the unnecessary 10-fold factor to extrapolate from a LOEL to a NOEL), a MADL of 2200 micrograms per day is calculated. This approach should be used to establish a scientifically more appropriate MADL for SO₂ that is still appropriately conservative.

Alternatively, a MADL could be derived based on a benchmark dose approach. The benchmark dose approach is currently permitted by the Proposition 65 regulations. Using a benchmark dose approach in order to predict a NOEL would lead to a MADL of 2200 micrograms per day or greater.

Study Selection

SO₂ was placed on the Proposition 65 list on July 29, 2011 as a developmental toxicant, but not as a male or female reproductive toxicant. According to the Proposition 65 regulations, the MADL shall be established considering the scientific basis for the listing. The regulations state:

“The determination of whether a level of exposure to a chemical known to the state to cause reproductive toxicity has no observable effect for purposes of Section 25249.10(c) of the Act shall be based on evidence and standards of comparable scientific validity to the evidence and standards which form the scientific basis for the listing of a chemical as known to the state to cause reproductive toxicity.”²

Since SO₂ was placed on the Proposition 65 list on the basis of its potential to cause developmental effects, the MADL for SO₂ must be based on a NOEL for developmental toxicity (as opposed to male or female reproductive toxicity). In addition, it is clear from the transcript of the July 12, 2011 meeting of the Developmental and Reproductive Toxicant Identification Committee (DARTIC) that there were only two endpoints of developmental toxicity (i.e., preterm birth and reduced fetal growth) that were the basis for the listing decision:

CHAIRPERSON BURK: Shall we split this discussion up into – are we safe in saying we probably won’t be discussing pregnancy loss and/or asthma and/or --- well, I don’t know, malformations. Does anybody think that’s – all right.

So, I think that, you know, the main two areas we’re going to talk about are preterm birth and the growth issues.³

Later in the transcript, it is also made clear by the DARTIC that it did not consider malformations, behavioral effects, childhood asthma, pregnancy loss or spontaneous abortions to be part of the basis for listing SO₂:

² Section 25801(a)

³ Transcript of July 12, 2011 Meeting of the Proposition 65 Developmental and Reproductive Toxicant Identification Committee, pp. 70-71.

CHAIRPERSON BURK: ... So I think we're back to preterm birth, and particularly to the fetal growth issues. So do we have any continuation to the preterm growth epidemiologist debate here?

I guess you'll agree to disagree kind of thing.

Okay. All right. Let's move on then to – actually, I want to make sure that we actually talked about all the other ones. And I think there's insufficient evidence for animal social behavior, the childhood asthma limited to prenatal exposure.

The congenital malformations, you know, there was a ton of data, but it seemed very inconsistent to me and not particularly plausible. Any comment on that, Carl?

COMMITTEE MEMBER KEEN: Well, I would concur. ... ⁴

CHAIRPERSON BURK: ... Also, even OEHHA staff does not find support for pregnancy loss or spontaneous abortions. So I don't think we need to discuss that one.

So, I think we'll talk about fetal growth ... ⁵

Thus, it is clear from the transcript that the basis for listing SO₂ as a developmental toxicant was preterm birth and reduced fetal growth and not other endpoints of developmental toxicity (e.g., malformations, behavioral effects, childhood asthma, pregnancy loss, spontaneous abortions).

We agree with OEHHA that the developmental toxicity study of inhaled SO₂ in mice, conducted by Murray et al. (the Murray et al. study⁶) is the “most sensitive study deemed to be of sufficient quality” for purposes of establishing a Proposition 65 MADL. As noted in the Proposed MADL Document, a statistically significant decrease in fetal body

⁴ Id., p. 84.

⁵ Id., p. 85.

⁶ Murray FJ, Schwetz BA, Crawford AA, Henck JW, Staples RE (1977) Teratogenic potential of sulfur dioxide and carbon monoxide in mice and rabbits. DOE Symposium Series 47:469-78.
Murray FJ, Schwetz BA, Crawford AA, Henck JW, Quest JF, Staples RE (1979) Embryotoxicity of inhaled sulfur dioxide and carbon monoxide in mice and rabbits. J Environ Sci Health C13(3):233-50.

weight was reported among the offspring of pregnant mice exposed to a nominal concentration of 25 ppm⁷ of SO₂ on days 6 through 15 of gestation compared to unexposed controls. However, a recent re-analysis of these data demonstrates that the decrease in fetal body weight reported at 25 ppm of SO₂ was slightly smaller than originally reported, and more importantly, it is not statistically significant. This information is presented in greater detail in the next section of this submission.

The purpose of the Murray et al. study was to evaluate the potential interaction of two common air pollutants: SO₂ and carbon monoxide. This study was conducted under a grant from the National Institute of Environmental Health Sciences (NIEHS). A separate developmental study of inhaled carbon monoxide alone was conducted in mice and rabbits under the same NIEHS grant in the same laboratory at approximately the same time as the Murray et al. study.⁸ The Murray et al. study was designed to evaluate whether SO₂ and carbon monoxide in combination would have an additive or synergistic effect on fetal development. The Murray et al. study was not specifically designed to identify the developmental toxicity NOEL for SO₂. There were three groups of mice in this study: (1) unexposed controls, (2) 25 ppm SO₂ alone, and (3) 25 ppm SO₂ and 250 ppm carbon monoxide in combination. Thus, only one exposure level of SO₂ (25 ppm) was studied in mice.

Evaluation of the fetal body weight data in the Murray et al. study indicates that 25 ppm SO₂ is the NOEL

A recent re-analysis by Exponent of the data from the Murray et al. study reveals that exposure of pregnant mice to 25 ppm of SO₂ did not produce a statistically significant decrease in fetal body weight as originally reported. Because the decrease in fetal body weight is not statistically significant, the 25 ppm of SO₂ exposed group in the Murray et al. study should be considered a NOEL for fetal body weight, not a LOEL.

⁷ While the nominal concentration was 25 ppm SO₂, the analytical concentration was 23.9±3.2 ppm SO₂. For purposes of simplicity, the concentration will be referred to as 25 ppm SO₂ in these comments. We do not dispute the use of 23.9 ppm in the Proposed MADL Document.

⁸ Schwetz BA, Smith FA, Leong BK, Staples RE (1979) Teratogenic potential of inhaled carbon monoxide in mice and rabbits. *Teratology* 19(3):385-92.

Several months ago, Dr. Ken Bogen of Exponent reviewed the tables in the Murray et al. study, and he theorized that the decreased fetal body weight among the 25 ppm of SO₂ group of mice was not statistically significant given the magnitude of the reported standard deviations. However, Dr. Bogen could not test his hypothesis without the individual litter data from the Murray et al. study. A successful effort was made to locate the original raw data from the Murray et al. study. All of the individual litter data worksheets, which were signed and dated, were located and identified. Based on the original individual litter data, Exponent was able to re-analyze the fetal body weight data for statistical significance. A copy of the Exponent summary report is appended herein. GMA will work with OEHHA to provide sufficient information to allow OEHHA to independently confirm the results of the Exponent statistical re-analysis.

Exponent found that the difference between the fetal body weight of the 25 ppm of SO₂ group and the control group is not statistically significant. The results of Exponent's evaluation of the data are compared with the results reported by Murray et al. (1979) in Table 1. Using an analysis of variance and a t-test, the *p* value (one-tailed) was 0.085. In order for the decrease in fetal body weight to be statistically significant, the *p* value must be less than 0.05. The Murray et al. study evaluated the fetal body weight data using an analysis of variance and Dunnett's test. Both the Exponent analysis and the original statistical analysis used a one-tailed test. The difference between the statistical methods does not explain the discrepancy between the statistical results.

Table 1. Fetal body weight data from the Murray et al. (1979) study and the re-analysis of the original litter data by Exponent (2012)

Parameter	Murray et al., 1979, Table 2		Exponent (2012) Re-analysis	
	0	25	0	25
Concentration of SO ₂ , ppm	0	25	0	25
Fetal Body Weight, g (Mean ± S.D.)	1.05±0.11	1.00±0.08*	1.05±0.11	1.01±0.08

* *p* < 0.05

In both the original study and in the Exponent re-analysis, fetal body weight was evaluated as the mean of the litter means. A small discrepancy in the mean fetal body weight was observed between the original reported value and the Exponent re-analysis. The reason for this difference is not apparent. It was confirmed that each mean fetal body weight for each litter was correctly calculated from the individual fetus data on the original individual litter work sheets. This suggests that perhaps some error, such as a transcription error, may have occurred in entering the data for the original statistical analysis. In the mid-70s, the statistical analysis was probably performed by entering data into a mainframe computer.

Based on the corrected mean value, the decrease in fetal body weight was slightly less than originally reported. The decrease in fetal body weight at 25 ppm of SO₂ was only 4% in the re-analysis, not 5% as originally reported.

The MADL should be based on the NOEL of 25 ppm of SO₂, eliminating the need for an additional default 10-fold factor

The MADL for SO₂ in the Proposed MADL Document is based on a forced estimate of the NOEL from the Murray et al. study. In this study, a small decrease in fetal body weight was reported in mice at 25 ppm, the only concentration of SO₂ evaluated in the Murray et al. study. Since this decrease was reported to be statistically significant (erroneously), the Proposed MADL Document regarded 25 ppm of SO₂ as a LOEL, not a NOEL, for fetal body weight. The Proposed MADL Document states, “Since adverse developmental effects were seen at the lowest dose used in this study, the LOEL is divided by 10 to establish a NOEL for purposes of assessment.” The Proposed MADL Document used the default 10-fold factor to estimate a NOEL from the LOEL of 25 ppm presumably because the regulations state:

“When data do not allow the determination of a NOEL, the lowest observed effect level in a study shall be divided by 10 to establish a NOEL for purposes of assessment.”⁹

⁹ Section 25803(a)(8)

The results of the Exponent re-analysis are important because they show that 25 ppm of SO₂ is more accurately considered the NOEL for fetal body weight in the Murray et al. study. Since it is now evident that the NOEL for fetal body weight is 25 ppm of SO₂, it is not scientifically appropriate to apply a 10-fold default factor, the default method for estimating a NOEL from a LOEL. In view of this new information, the MADL for SO₂ should be 2200 micrograms per day because the default 10-factor should not be applied to the NOEL.

Even if 25 ppm of SO₂ had been confirmed to produce a statistically significant decrease in fetal body weight in the Murray et al. study, it would still be scientifically more appropriate to estimate a NOEL from the “LOEL” using a data-driven factor. In the Proposed MADL Document, the MADL is based on a NOEL derived by applying the default 10-fold factor to the “LOEL”. However, it is appropriate to use the default factor only “in the absence of principles or assumptions scientifically more appropriate based upon the available data.”¹⁰ Even if 25 ppm of SO₂ had been confirmed to be the LOEL, there are sufficient data to estimate the NOEL without relying on the default 10-fold factor. Fortunately, it is not necessary to estimate the NOEL since there is clear evidence that 25 ppm of SO₂ is the NOEL for decreased fetal body weight.

Even before re-analyzing the data from the Murray et al. study, it was apparent the decrease in fetal body weight at 25 ppm of SO₂ was very close to the limit of statistical significance. A 5% decrease in fetal body weight is about the smallest decrease in fetal body weight that can be shown to be statistically significant in a conventional rodent developmental toxicity study with normal group sizes of 20-25 litters. Even if 25 ppm of SO₂ had been a LOEL (which it is not), there is a strong indication that the NOEL must be very close to 25 ppm of SO₂.

Applying the default 10-fold factor to a “LOEL” of 25 ppm of SO₂ to predict a NOEL results in an estimated NOEL of 2.5 ppm of SO₂. This means that the default approach estimates that an exposure level of 2.5 ppm of SO₂ is predicted to cause about a 5% decrease in fetal body weight. This estimate is inconsistent with the results of the Murray

¹⁰ Section 25803(a)

et al. study, which upon re-analysis now demonstrates a 4% decrease in fetal/pup body weight at 25 ppm of SO₂. It is also inconsistent with the results of another developmental toxicity study of inhaled SO₂ in mice that observed a 4% decrease in Day 1 pup body weight at 32 ppm of SO₂ (Singh, 1989)¹¹. Even without the statistical analysis, it is clear that 25 ppm of SO₂ is closer to a NOEL than a LOEL.

The MADL could be established by using a benchmark dose approach

As an alternative to using the NOEL, it may be scientifically appropriate to use a benchmark dose (BMD) to establish a MADL for SO₂. Even if 25 ppm of SO₂ had been a LOEL, it is clear from the regulations that the default 10-fold factor to estimate a NOEL from a LOEL is to be used only when a scientifically more appropriate approach is not available. The default assumptions in the Proposition 65 regulations are prefaced with the following sentence:

“In the absence of principles or assumptions scientifically more appropriate based upon the available data, the following default principles and assumptions shall apply in any such assessment.”¹²

Fortunately, based on the data in the Murray et al. study, there are scientifically more appropriate principles and assumptions to be applied than dividing the “LOEL” by the default factor of 10 to estimate a NOEL. As an alternative using the NOEL/LOEL approach, the Proposition 65 regulations now specifically identify the benchmark dose methodology as a generally accepted scientific methodology for estimating a NOEL.

“The NOEL shall be the highest exposure level which results in no observable reproductive effect expressed in milligrams of chemical per kilogram of body weight per day. This may be the no observed effect level in a scientific study *or, alternatively, may be calculated by means of a generally accepted scientific methodology such as the benchmark dose methodology.*”¹³ [emphasis added]

¹¹ Singh J (1989). Neonatal development altered by maternal sulfur dioxide exposure. *Neurotoxicology* 10(3): 523-7.

¹² Section 25803(a)

¹³ Section 25803(a)(2)

The benchmark dose approach is an alternative to the NOEL/LOEL approach that has been used for many years in dose-response assessment. The development of this approach has been pursued because of recognized limitations in the [NOEL/LOEL](#) approach.

If there is a minimal level of change in the endpoint that is generally presumed to be biologically significant (for example, a change of 5% or 10%), then that amount of change can be used to define the benchmark dose. For example, the benchmark dose for fetal body weight in developmental toxicity studies is typically expressed as the BMD05 or BMD10 to indicate it is an estimate of the dose that produces a 5% or 10% decrease in fetal body weight. Comparison of the BMD with the No Observed Adverse Effect Level (NOAEL) for a large number of developmental toxicity data sets indicated a benchmark dose response (BMR) in the range of 5 to 10 % resulted in a BMD that was on average similar to the NOAEL.¹⁴ This was described more fully in a US EPA report:

“The fact that a BMD corresponds to a specified level of change in response to an adverse effect (for quantal data, generally 1 percent to 10 percent increased risk, as discussed earlier) and a NOAEL ostensibly corresponds to an experimental dose with no adverse effect does not imply that NOAELs will necessarily be smaller than BMDs (and consequently that larger uncertainty factors may be appropriate for BMDs). First, a BMD is defined as a statistical lower limit, which introduces an element of conservatism in its definition. Second, one cannot conclude that no adverse effects are possible at a NOAEL or that effects will necessarily be observed at the BMD. The BMD corresponding to an extra risk of 1 percent was smaller than the corresponding NOAEL for each of 10 data sets studied by Gaylor (1989). Among five sets of quantal data studied by Crump

¹⁴ Allen, B. C.; Kavlock, R. J.; Kimmel, C. A.; Faustman, E. M. (1994a) Dose-response assessment for developmental toxicity: II. Comparison of generic benchmark dose estimates with NOAELs. *Fund. Appl. Toxicol.* 23: 487-495.

Allen, B. C.; Kavlock, R. J.; Kimmel, C. A.; Faustman, E. M. (1994b) Dose-response assessment for developmental toxicity: III. Statistical models. *Fund. Appl. Toxicol.* 23: 496-509.

Faustman, E.M.; Allen, B.C.; Kavlock, R.J.; Kimmel, C.A. (1994) Dose-response assessment for developmental toxicity: I. Characterization of data base and determination of NOAELs. *Fund. Appl. Toxicol.* 23: 478-486.

(1984), the BMD corresponding to an extra risk of 1 percent was larger than the NOAEL in one case by a factor of 1.4, and smaller than the NOAEL in three cases by factors ranging from 1.1 to 2.6 (one data set did not define a NOAEL). However, it is unclear whether the data sets used in these studies are typical of those to which the BMD method would be applied if the method is used routinely. In a comparison study of a large number of developmental toxicity data sets (Allen et al., 1994a, b; Faustman et al., 1994), a BMD corresponding to an extra risk of 5 percent was on average similar to the NOAEL when expressed as probability of response per litter.”¹⁵

The reason why a BMD05 is considered a practical alternative to a NOEL for fetal body weight is, in a conventional developmental toxicity study with group sizes of 20-25 litters, a 5% decrease in fetal body weight is typically at the border of the ability of statistical methods to detect a statistically significant decrease in fetal body weight. In other words, even though the BMD05 is a theoretical estimate of the dose that produces a 5% decrease in fetal body weight, the BMD05 corresponds closely with the NOEL for this parameter. For this reason, the BMD05 is a scientifically appropriate estimate of a study’s NOEL for fetal body weight.

Use of benchmark dose methodology typically involves fitting mathematical models to dose-response data and using the different results to select a BMD that is associated with a predetermined BMR, such as a 5% or 10% decrease in fetal body weight. In the Murray et al. study, only one exposed group (i.e., the 25 ppm SO₂ group) is available for estimating a BMD. Coincidentally, the only SO₂ (alone) exposed group exhibited a 4% decrease in fetal body weight, which is very close to a target benchmark response of 5%. So, although there are not multiple dose groups with which to model a BMD05, the actual response at 25 ppm of SO₂ in the Murray et al. study was a 4% decrease in fetal body weight, which is very close to a 5% decrease. In other words, the only concentration of SO₂ evaluated in the Murray et al. study just happens to provide direct evidence that exposure to 25 ppm SO₂ is very close to a BMD05.

¹⁵ Crump K, Allen B, Faustman E (1995) The use of the benchmark dose response in health risk assessment. EPA/630/R-94/007 <http://www.epa.gov/raf/publications/pdfs/BENCHMARK.PDF>

A BMD05 for fetal body weight may be directly estimated from the Murray et al. data. Confidence in a BMD05 of 25 ppm of SO₂ derived from the Murray et al. study in mice is enhanced by the results of the Singh (1989) study. As noted in the Proposed MADL Document, another inhalation developmental toxicity study of SO₂ by Singh in CD-1 mice demonstrated at 32 ppm of SO₂ a reduction in pup body weight on day 1 of birth comparable in magnitude to the decreased fetal body weight at 25 ppm reported by Murray et al. In fact, exposures to 32 and 65 ppm of SO₂ in the Singh (1989) produced a 4% and 11% decrease, respectively, in pup body weight. This result provides additional confidence that an accurate estimate of the BMD05 for fetal body weight is no less than 25 ppm.

Alternatively, the fetal body weight data from the Murray et al. study may be combined with the data from the Singh (1989) study to estimate a BMD05, and this approach is expected to produce a BMD05 greater than 25 ppm of SO₂. This approach has the advantage that a BMD05 could be estimated from three dose levels instead of just one. However, it is recognized that there are some differences in the experimental design between the two studies. These experimental design differences do not pose an insurmountable challenge for purposes of estimating a BMD from the combined data. For example, Murray et al. exposed pregnant mice to SO₂ for 7 hours per day, whereas Singh (1989) exposed pregnant mice for 24 hours per day. However, the exposures in both studies could be expressed in common terms such as milligrams per kilogram of body weight per day.

Some might contend that it is more appropriate to use a benchmark dose level (BMDL) than a BMD for estimating a NOEL for purposes of Proposition 65. The BMDL is a more conservative metric because it represents a lower confidence limit of the estimated BMD. Confidence limits express the uncertainty in a parameter estimate that is due to sampling and/or experimental error. A lower confidence limit is placed on the BMD to obtain a benchmark dose level (BMDL) that assures with high confidence (e.g., 95%) that the BMR is not exceeded. Yet, the uncertainty surrounding a BMD05 estimated from the Murray et al. study is small, given the fact that it is based on a dose group that produced a

4% decrease in fetal body weight and given the confirmatory data from the Singh study. In addition, US EPA and other regulatory agencies generally use a BMD10 rather than a BMD05. As such, the use of the BMD05 in deriving a MADL is conservative in itself. Therefore, we believe that the BMD05 provides a conservative estimate of the NOEL in the Murray et al. study for the purpose of establishing a Proposition 65 MADL. For example, using a conservative estimate of 25 ppm of SO₂ for the BMD05 would result in a MADL of 2200 micrograms/day, which is identical to the MADL derived from the NOEL of 25 ppm of SO₂.

In summary, a MADL for SO₂ of at least 2200 micrograms/day may be derived using a benchmark dose approach. With the exception of the default 10-fold factor to estimate a NOEL from the “LOEL”, this approach employed exactly the same assumptions and calculations used in the Proposed MADL Document. In the case of SO₂, a MADL derived using the benchmark dose approach is scientifically more appropriate and accurate than the MADL derived by dividing the “LOEL” by the default 10-fold factor in the Proposed MADL Document.

Summary

The NOEL for fetal body weight in the most sensitive developmental toxicity study is 25 ppm of SO₂. This value results in an MADL of 2200 micrograms per day. Additionally, using the benchmark dose approach also gives an MADL of 2200 micrograms per day, if not greater. We recommend an MADL of 2200 micrograms per day be established based on the best scientifically appropriate methods.

III. Food Chemistry and Sulfur Dioxide (SO₂)

Introduction to Food Chemistry Issues

The following comments on the food chemistry aspects of the Proposed MADL are directed specifically to the Proposed MADL's "Initial Statement of Reasons (ISOR)" in the two-paragraph section in Appendix A entitled, "Applicability of the MADL" (page 7). While these comments are not directly being made on the "Interpretive Guideline No. 2012-02, Consumption of Sulfur Dioxide in Dried Fruits" [the IG] (because OEHHA is not currently seeking comments on that document), we are providing these comments for purposes of clarification and correction of various statements, concepts and discussions in the IG. We furthermore urge OEHHA to include scientific explanations and facts we are providing here in the "Final Statement of Reasons" and the Final MADL considerations.

By way of introduction, the amount of SO₂ in food and food ingredients has been a common commercial and regulatory specification since the early part of the last century. Initially this specification was adopted to deter economic adulteration of foods, such as the application of sulfites to fresh meat to provide an appearance of freshness. Because of the limitations of existing analytical methods to differentiate between sulfite species, a single measurable end-point for all sulfites was adopted by government and industry.¹⁶ As far back as the mid-1980s, the level of sulfites in foods was determined by the U.S. Food and Drug Administration (FDA) to be the level of sulfur dioxide quantified by **existing methods that convert all sulfites to SO₂ for analytical purposes**. At that time FDA's concern was with potential allergenic reactions within a small population of sulfite-sensitive, asthmatic individuals, and FDA's use of a "SO₂" specification was based on the chemistry and analytical methodology discussed below.

"Molecular Sulfur Dioxide" Does Not Exist in Foods

The most fundamental scientific disagreement that we have with OEHHA's evaluation of the complex food chemistry of products that have been treated with SO₂ gas or various

¹⁶ Taylor, S.L., Higley, N.A., and Bush, R.K. 1986. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity and hypersensitivity. *Adv. Food Res.* 30: 1-76.

sulfite food additives is that **“molecular SO₂” does not exist as such in foods and beverages**. This fact is well supported in published reviews by leading experts.¹⁷ It is well recognized in the literature that there is extensive reactivity of SO₂ and sulfites with numerous classes of compounds in foods and beverages. Sulfur dioxide exists as the chemical species **“hydrated sulfur dioxide” (SO₂ •H₂O)** in equilibrium with sulfurous acid in foods and beverages, and there is at most only a negligible quantity of SO₂ that can exist as SO₂ gas in the headspace above foods and beverages. “Dissolved” SO₂ gas simply does not exist in aqueous solution or in foods; when it is present, it off gasses into the product’s headspace, leading to only a negligible, perhaps immeasurable consumer exposure to the gaseous form. Therefore, since there really is no “molecular SO₂” in foods and beverages except for negligible amounts in the headspace, we conclude that there is no exposure under Proposition 65 to “molecular SO₂” in foods and beverages. We will examine the evidence for this conclusion in the following sections on the detailed food chemistry of sulfur dioxide and sulfites.

The Taylor et al. comprehensive review of sulfites in foods provides a critical appraisal of sulfite analytical methodology and also addresses some key misconceptions about the actual occurrence and nomenclature of these chemical species in foods.¹⁸ Excerpts from their review (quoted below) will serve to inform the debate about these chemical complexities and common misconceptions (excerpted from pages 18-21):

“Sulfites exist in foods as sulfurous acid, inorganic sulfites, and a variety of forms of combined sulfites. Complex equilibria dependent on a number of factors control the amount of sulfite in each of these states...Methods for the measurement of free SO₂ are aimed at detection of undissociated sulfurous acid, bisulfite ions, and sulfite ions. Methods for the measurement of total SO₂ are

¹⁷ Wedzicha, B.L., *Chemistry of Sulphur Dioxide in Foods*, Elsevier Applied Science Publishers, London, 1984.

Taylor, S.L., Higley, N.A., and Bush, R.K. 1986. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity and hypersensitivity. *Adv. Food Res.* 30: 1-76.

Wedzicha, B.L. 1992. Chemistry of sulphiting agents in food. *Food Add. Contam.* 9: 449-459.

Wedzicha, B.L. 2000. “Effects of Sulfur Dioxide on Food Quality,” in *Food Shelf Life Stability: Chemical, Biochemical, and Microbiological Changes*, ed. David S. Robison and N.A. Michael Eslin, CRC Press LLC, Boca Raton, FL, 30 pages.

¹⁸ Taylor, S.L., Higley, N.A., and Bush, R.K. 1986. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity and hypersensitivity. *Adv. Food Res.* 30: 1-76.

aimed at detection of these substances plus some of the combined forms of the sulfites. Generally, the methods for analysis of totalSO₂ can be subdivided into two groups: those in which the combined SO₂ is liberated by distillation from acid, and those in which the combined SO₂ is liberated by treatment of an extract with excess alkali. SO₂ will not be liberated from all forms of combined sulfite by either of these treatments; some combined sulfites are quite stable. The levels of combined sulfites are not included in some methods of analysis as a distinct determination. They are often calculated from the differences between total SO₂ and free SO₂, and thus are underestimates representing only the dissociable forms of combined sulfites.”

“For unknown reasons that probably date back many years, the measurements of free and total sulfite residues in foods are referred to as free and total SO₂ analyses. The reason probably relates to the release of SO₂ under the conditions of the assay. **However, SO₂ is not the form that actually exists in foods.** The free SO₂ methods are actually detecting residues of free inorganic sulfite salts. It would be preferable to refer to them as assays of free sulfite (or free sulfite as SO₂) rather than free SO₂. The total SO₂ methods are detecting the free sulfite residues as well as some of the combined forms of sulfite. The combustion method described by Wedzicha et al.¹⁹ may detect most of the combined forms of sulfite. These methods should be referred to as assays of total sulfite (or total sulfite as SO₂) rather than total SO₂, although the use of the adjective total may be misleading, since not all forms of combined sulfites can be detected with these assays.” [emphasis added]

“Considerable data exist in the literature on residual SO₂ levels in foods... However, for several reasons, we have some reservations about these data. Many methods exist for the measurement of residual sulfite levels in foods, and correlations between the various methods have not been established for most

¹⁹ Wedzicha, B.L., Lamikanra, O., Herrera, J.C. and Panahi, S. 1984. Recent developments in the understanding of the chemistry of sulphur(IV) oxospecies in dehydrated vegetables. Food Chem. 15: 141-155.

foods. Therefore, it is difficult to evaluate the validity of some of the published residue data. Some of the methods used to generate these residue data have subsequently been shown to give erroneous results. Second, very little residue data are available for sulfited foods obtained from the marketplace. Much of the available data were obtained from products sampled immediately after processing. Therefore, the effects of storage and any differences with standard, present-day commercial practices have not been taken into account. Much of the available residue data are from fairly old studies, and treatment conditions have probably changed in the intervening years. As mentioned previously, the effects of preparation on residual sulfite levels have essentially been ignored in previous work.”

“Sulfur dioxide dissolves readily in water-producing sulfurous acid, H_2SO_3 . On treatment with alkali, sulfurous acid yields sulfites, bisulfites, and metabisulfites. These inorganic forms of sulfites are in equilibrium with one another in aqueous solutions and the concentration of each species is dependent on pH. At high pHs, SO_3^{2-} is the predominant species, while at very low pHs, H_2SO_3 predominates. At intermediate pHs, HSO_3^- predominates, reaching a maximum concentration at pH 4.0. SO_2 can be evolved from H_2SO_3 , but only at acid pHs. **Note that no SO_2 can be evolved from a solution until the pH drops to pH 4.0 or below.**” [emphasis added]

As stated elsewhere in these comments, it is critical for the purposes of Proposition 65 compliance that food manufacturers accurately determine the levels of listed chemicals in foods. Furthermore, we agree with OEHHA’s conclusion that only the listed substance, sulfur dioxide, which is a gaseous molecule, is subject to the law’s regulatory requirements, and that the regulations do not apply to sulfites, bisulfites or metabisulfites (as stated in your Initial Statement of Reasons). We would also urge OEHHA to add to this list of non-regulated substances (as pointed out below) both “sulfurous acid” and “hydrated sulfur dioxide,” which are also known to be involved in the complex equilibria of foods treated with sulfur dioxide and sulfites.

However, we do not agree with OEHHA that the “*Molecular SO₂ Calculated from Free SO₂ (ppm)*” levels that are estimated and reported for various dried fruit products in Table 1 of the IG represent the true concentration of the listed substance “Sulfur Dioxide” in these products. In fact, owing to the long-held scientific opinions of experts (quoted herein) in the field of sulfur dioxide/sulfites that gaseous, molecular sulfur dioxide does not exist as that specific molecule within foods, we believe that there are essentially no exposures to this listed chemical from foods. In addition, the harsh treatment methods (using heat, acid digestion, alkalization, etc.) that are required to try to estimate various quadrivalent S(IV) oxospecies in food, such as in the Monier-Williams and related methods described later in these comments, give us estimates of only “apparent SO₂” or “SO₂ equivalents” but not estimates of a food product’s concentration of molecular SO₂, which we believe does not exist in foods.

As noted in OEHHA’s Hazard Identification Document, “Evidence on the Developmental and Reproductive Toxicity of Sulfur Dioxide,” released for public comment in February 2011 (see B.1. Chemical Structure and Characteristics, page 17):

“SO₂ in contact with water readily produces sulfurous acid (Cosmetic Ingredient Review Expert Panel (CIREP, 2003). At a pH of 7.4 and temperature of 37°C (physiological conditions), a mixture of sulfite ions and bisulfate ions will predominate. **More acidic conditions liberate SO₂ vapor.** With more alkaline conditions, sulfites, bisulfites and metabisulfites are produced.” [emphasis added]

Using the “Total SO₂” or “Free SO₂” analytical measurements, and then trying to estimate the amount of the listed chemical SO₂ by selecting the food’s pH and estimating the “molecular SO₂” level from the curves in Figure 1 in the IG document, does not represent the true level of SO₂ in any food product. In fact, this technique grossly overestimates the SO₂ content of every food tested, and essentially gives a worst-case scenario of all the combined S(IV) chemical species found in the food.

In Figure 1 in the IG document, the curve on the far left (starting at pH 0) is actually the dissociation curve of sulfurous acid or SO₂ •H₂O (“hydrated SO₂”), not of “molecular

SO₂.” We believe that OEHHA mislabeled this curve as the “SO₂” dissociation curve, when in reality it is the dissociation curve of sulfurous acid or SO₂ •H₂O (“hydrated SO₂”). At the pK_a = 1.81, there exists in solution of a liquid food/beverage or in the aqueous phase of a solid food only two chemical species:

(1) 50% SO₂ •H₂O (“hydrated SO₂”), not molecular SO₂ gas, in the leftmost descending curve; and

(2) 50% HSO₃⁻ (bisulfite ion) in the curve in the middle of Figure 1.

Therefore, it is not scientifically accurate to call the concentration of the species at the pH of a food below about 4.0 the actual amount of “molecular SO₂” in the food. But this is the method that OEHHA erroneously uses in the IG to “estimate” the amount of “molecular SO₂” in various dried fruits products entered into the rightmost column of Table 1, “Molecular SO₂ Calculated from Free SO₂ (ppm)” and subsequently to calculate the “Estimated Exposure to Molecular SO₂ in Fruit (µg/day)” in the rightmost column of Table 2.

It is pointed out in numerous reviews that SO₂ gas in foods exists only in the headspace above the food and is subsequently released into the surrounding atmosphere.

Consequently, consumers may be exposed to negligible, perhaps immeasurable amounts of off-gassed SO₂ during food preparation or consumption, but they will not be exposed to molecular SO₂ from the consumption of the food product itself.

General Uses of Sulfur Dioxide Gas and Sulfites in Food Processing

As reviewed by Taylor et al. (1986), sulfiting agents have a long history of use as food ingredients and additives. The term “sulfiting agents” refers to sulfur dioxide (SO₂) and several forms of inorganic sulfite that liberate SO₂ under the varied conditions of use. In addition, naturally occurring sulfites are present in many foods; the yeast cultures used in the fermentation of wines and beers naturally produce a portion of the sulfites found in these products.

Sulfiting agents are added to foods for many important technical purposes, including the control of enzymatic and nonenzymatic browning, antimicrobial action, antioxidant and reducing agent uses, bleaching agent uses and a variety of processing aid uses, including several secondary uses such as a pH control agent and stabilizing agent. In many products, the sulfites serve more than one purpose. Sulfiting agents are currently used in a wide variety of food products, and wide variations in treatment modes and levels for particular products are known to occur in the food industry globally. Sulfur dioxide (SO₂), potassium bisulfite (KHSO₃), potassium metabisulfite (K₂S₂O₅), sodium bisulfite (NaHSO₃), sodium metabisulfites (Na₂S₂O₅), and sodium sulfite (Na₂SO₃) are approved by the FDA for various uses. In addition to their use as food additives, it must be remembered that sulfites can also occur naturally in foods, and foods contain a variety of sulfur-containing compounds, including the sulfur amino acids, sulfates, sulfites and sulfides. These sulfur-containing compounds are interconvertible in some food systems that possess the appropriate enzymes.

Introduction to the Food Chemistry of Sulfur Dioxide and Sulfites

There have been several major reviews of the extensive literature on the food chemical aspects of SO₂ and its salts going back almost 30 years.²⁰ The following comments are largely drawn from these expert reviews and will serve to inform the debate over the complexities inherent in the food chemistry of SO₂ and its numerous related chemicals species.

Sulfur dioxide (SO₂) gas and various sulfite salts have been used as direct additives in food preservation since ancient times. They are still regarded as indispensable in many

²⁰ Wedzicha, B.L., *Chemistry of Sulphur Dioxide in Foods*, Elsevier Applied Science Publishers, London, 1984.

Taylor, S.L., Higley, N.A., and Bush, R.K. 1986. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity and hypersensitivity. *Adv. Food Res.*30: 1-76.

Wedzicha, B.L. 1992. Chemistry of sulphiting agents in food. *Food Add. Contam.* 9: 449-459.

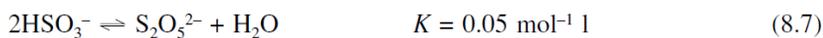
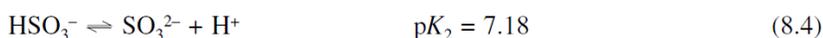
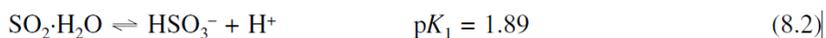
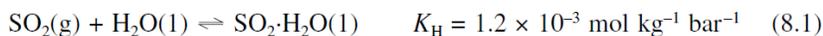
Wedzicha, B.L. 2000. "Effects of Sulfur Dioxide on Food Quality," in *Food Shelf Life Stability: Chemical, Biochemical, and Microbiological Changes*, ed. David S. Robison and N.A. Michael Eslin, CRC Press LLC, Boca Raton, FL, 30 pages.

antimicrobial applications and unique in their ability to control most types of chemical (enzymic and nonenzymic) food spoilage, and the use of these preservatives is still approved in most countries of the world. Sulfites are unusually reactive among food additives; the concentration in a given food at the time of sale is often half, or less, of the amount added at the time of production. Such reactivity is both specific to the intended action of the additive and non-specific as a result of its broad spectrum of reactivity. The specific reactions of sulfites with key intermediates in chemical spoilage are now being used to provide a fundamental understanding of those spoilage mechanisms. Moreover, the chemical reactivity of sulfite can be used to probe for the nature of the aqueous phase in food as a medium for chemical reactions, particularly under non-ideal conditions, e.g., high ionic strength, low water activity.

Chemical Nature of Sulfur Dioxide Gas and Related Species

The terms *sulfur dioxide* or *sulfite(s)* refer to oxospecies of sulfur in the quadrivalent oxidation state (IV). They are all derived by the dissociation of the so-called sulfurous acid H_2SO_3 . Despite the wide use of the term *sulfurous acid* in the chemical literature, it is acknowledged that this species does not exist as such. Thus, it has become conventional to represent the weak, dibasic sulfurous acid as $\text{SO}_2 \bullet \text{H}_2\text{O}$ (“hydrated SO_2 ”), while recognizing that it has the properties of H_2SO_3 . The following equilibria need to be taken into account in the discussion of the reactivity of sulfites in food:²¹

²¹ Wedzicha, B.L. 2000. “Effects of Sulfur Dioxide on Food Quality,” in Food Shelf Life Stability: Chemical, Biochemical, and Microbiological Changes, ed. David S. Robison and N.A. Michael Eslin, CRC Press LLC, Boca Raton, FL, 30 pages.



where the values of all equilibrium constants given for the specific reactions are at 25°C and infinite dilution, K_{H} is Henry's constant, and M^{n+} represents a metal ion.

The fate of sulfites in foods is thus an extremely complex situation. The combination with organic constituents, the equilibrium between the various inorganic forms, the volatilization of SO_2 and the oxidation to sulfate can all be important reactions.

In the normal pH range of food, pH 3 to 6, the principal species is bisulfite anion (HSO_3^-) in equilibrium with small but pH-sensitive amounts of $\text{SO}_2 \cdot \text{H}_2\text{O}$ and sulfite anion (SO_3^{2-}). These minor species are responsible for the preservative action and chemical reactivity of the additive. However, it is important to appreciate that, in some instances, the $\text{p}K$ values of $\text{SO}_2 \cdot \text{H}_2\text{O}$ are sensitive to the composition of the medium, other than its pH. Thus, the correct $\text{p}K$ values of $\text{SO}_2 \cdot \text{H}_2\text{O}$ in a given food situation are subject to some debate. There is no simple rule to estimate these, and they are likely to be dependent on the food matrix itself.

It can be seen that the state of SO_2 or sulfite in food is complex, but all the forms that have been identified thus far are readily and rapidly interconvertible. Regardless of the chemical form in which S(IV) is added to food (e.g., gaseous SO_2 , sodium or potassium metabisulfites), the actual composition of this preservative depends on the pH of the

food, the concentration of S(IV), the ionic strength and the presence of non-electrolytes.²² During analysis, all these forms of S(IV) are converted either to SO₂ (as in the Monier-Williams distillation technique, or its adaptations, see below) or to some other well-defined species (e.g., SO₃²⁻ for ion chromatography). In view of the complex speciation of the additive in any given food situation, the convention adopted in the literature refers to the mixture of sulfur(IV) oxospecies, in all forms which are readily converted to SO₂ on acidification, as S(IV).

One of the most recent and definitive food chemistry textbooks²³ provides the following reaction scheme to describe the reactions that occur in foods and beverages:

368

Chapter 9

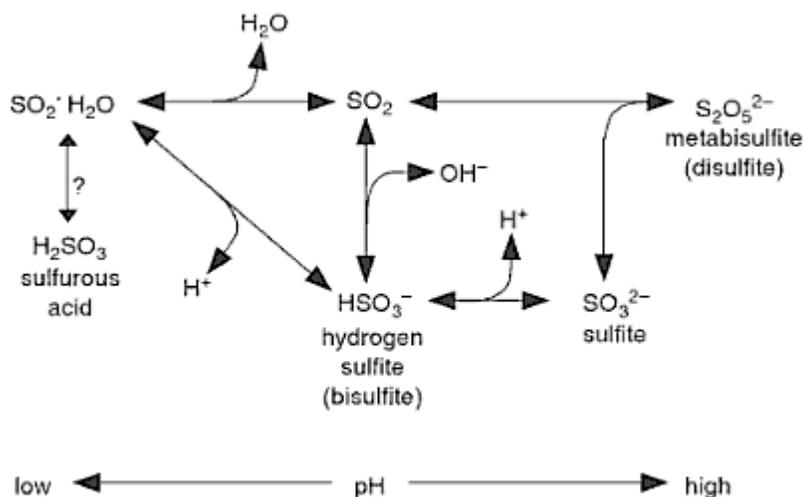


Figure 9.1 Structural relationships between sulfur dioxide-generating compounds.

It is important to point out in this reaction scheme that sulfurous acid is in equilibrium with SO₂ • H₂O (“hydrated SO₂”), which upon release of a molecule of H₂O, allows the

²² Wedzicha, B.L. 2000. “Effects of Sulfur Dioxide on Food Quality,” in *Food Shelf Life Stability: Chemical, Biochemical, and Microbiological Changes*, ed. David S. Robison and N.A. Michael Eslin, CRC Press LLC, Boca Raton, FL, 30 pages.

²³ Coultate, T.P. 2009. *Food: The Chemistry of its Components*, 5th Edition, Chapter 9, “Preservatives - Sulfur Dioxide,” Royal Society of Chemistry Publishing, Cambridge, UK, pp. 367-369, accessible at *Google Books*.

release of SO₂ either into the vapor phase as a gas or to further equilibrations to form hydrogen sulfite (bisulfite) anion or metabisulfite anion.

Chemical Reactivity of S(IV): Nucleophilic Reactions

Quadrivalent sulfur(IV) [S(IV)] oxospecies (oxoanion salts collectively referred to as sulfites) show two distinct types of reactivity in foods, leading to a complex mixture of S(IV) products that exhibit a variety of chemical properties. Some of these substances are labile and can be lost during isolation and separations during analytical procedures. The sulfite anion (SO₃²⁻) itself is an excellent nucleophile, whereas all the S(IV) species behave as reducing agents. The main reason for reactions between S(IV) and food components is the nucleophilic reactivity of SO₃²⁻, leading to the formation of C–S and S–S covalent bonds. Sulfite ion is one of the best nucleophiles available, with reactivity similar to that of the thiolate ion, acting both as a carbon- and a sulfur-nucleophile. Taylor et al. (1986)²⁴ extensively describe the wide range of reactions of sulfites with numerous common food constituents, including carbonyl compounds, reducing sugars, proteins and amino acids, vitamins, nucleic acids and nucleotides, anthocyanin pigments and fatty acids.

This ability to readily undergo nucleophilic reactions will now be considered in relation to the two most important functional attributes of S(IV) additives in food, that of: (1) as an antimicrobial agent; and (2) as an inhibitor of nonenzymic browning:

(1) Chemistry of the Antimicrobial Behavior of S(IV)

It has long been recognized that “undissociatedSO₂•H₂O” is the effective antimicrobial species in S(IV) mixtures, and the general use of the term “molecular SO₂” in this context is remarkably well informed, at a time when few are still aware of the unlikely existence of sulfurous acid as H₂SO₃. Perhaps the best known interaction between components of living systems and S(IV)

²⁴ Taylor, S.L., Higley, N.A., and Bush, R.K. 1986. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity and hypersensitivity. *Adv. Food Res.* 30: 1-76.

compounds, and of considerable significance in foods, is the addition of HSO_3^- to carbonyl groups forming adducts of varying stability. These adducts become progressively labile (are formed and decomposed more rapidly) as pH is increased. Outside this range of pH, hydroxysulfonates are less stable; as pH is reduced to below 3, the increasing conversion of S(IV) to SO_2 results in the apparent value of K passing through a minimum at around pH 2, although the rates of formation and decomposition of the adducts are very slow. On the other hand, they decompose rapidly at $\text{pH} > 7$.

(2) Inhibition of Nonenzymic Browning

The term nonenzymic (or nonenzymatic) browning is synonymous with the Maillard Browning Reaction, i.e., the reaction between reducing sugars and amino acids, peptides and proteins in the presence of heat. A discussion of this important food reaction is beyond the scope of these comments. However, in general, the major products of the irreversible combination of S(IV) compounds with food components are hydroxysulfonates and other products, and these reactions serve to inhibit the extent of nonenzymic browning reactions by decreasing the amount of reducing sugars available to react to produce initial and subsequent browning reaction products. These reactions also serve to block the formation of the characteristic brown pigments. In food dehydration, the additives are used to prevent spoilage in the intermediate moisture phase of the process, at which stage the rate of browning is at a maximum, and to protect the dehydrated food from browning while in storage. The effect of S(IV) compounds is to delay the onset of browning, but, once it commences, browning continues at the same rate in sulfited and unsulfited systems.

“Free” and “Bound” S(IV) Compounds

The classification of S(IV) in foods into *free* and *bound* S(IV) compounds is well known and referred to often in the food industry, but there are many instances where its significance is poorly understood. “Bound” S(IV) is sometimes referred to as *reversibly*

bound. “Free” S(IV) is the term used to describe the additive present in the form of SO₂ or any S(IV) species (e.g., SO₃²⁻, S₂O₅²⁻), which are converted rapidly to SO₂ gas upon acidifying. This term is, therefore, synonymous with the more accurate use (in the chemical sense) of the term S(IV). Bound S(IV), which is now regarded as mostly in the form of hydroxysulfonates (i.e., carbonyl-S(IV) adducts formed from reactions with aldehydes, ketones, reducing sugars and proteins), was defined originally in terms of the different stability and the rates of formation and decomposition of these products. Thus, bound S(IV) is the amount of the additive that is converted to the free form by raising the pH of a sample to at least pH 10, whereas free S(IV) is usually analyzed at pH ~ 2, under which conditions hydroxysulfonates are most stable. In general, the major products of the irreversible combination of S(IV) with food components are organic sulfonates, most often formed as a result of the inhibition of nonenzymic browning reactions.

Hydroxysulfonate dissociation constants vary for a wide range of carbonyl compounds, representing the extremes of stability normally encountered for food components. The reaction product for a simple aldehyde, e.g., acetaldehyde, is a very stable adduct within the pH range of most foods (pH 3-7). On the other hand, since the carbonyl group of reducing sugars exists in equilibrium with cyclic structures, the values of their hydroxysulfonate dissociation constants depend on the proportion of acyclic form present. Thus, the glucose and fructose hydroxysulfonates are more unstable adducts.

An equilibrium always exists between the free and bound forms of the sulfites, although some of these reactions are virtually irreversible, while others are more readily reversible.²⁵ Pizzoferrato et al.²⁶ make the clear distinction between free and bound sulfite and how their newly reported indirect photometric-HPLC avoids interference issues:

²⁵ Wedzicha, B.L., Lamikanra, O., Herrera, J.C. and Panahi, S . 1984. Recent developments in the understanding of the chemistry of sulphur(IV) oxospecies in dehydrated vegetables. *Food Chem.* 15: 141-155.

²⁶ Pizzoferrato, L., Di Lullo, G. and Quattrucci, E. 1998. Determination of free, bound and total sulphites in foods by indirect photometry-HPLC. *Food Chem.* 63: 275-279.

“...the former refers to all the species that may rapidly and quantitatively be converted to SO₂, thereby acidifying a treated food; the latter represents hydroxysulphonate adducts formed by reaction of carbonyl groups with HSO₃⁻ ... Comparing the two methodologies, bound form levels evaluated by the Monier-Williams procedure after heating, are confirmed to be affected by interferences due to co-distilled volatile anions. As a consequence, total sulphite, calculated as the sum of free and bound fractions, are overestimated by the Monier-Williams method. This over-estimation of the sulphite levels in foods can be avoided by using a separative method of analysis such as the proposed HPLC methods that, unlike the Monier-Williams method, avoids the potential interference of volatile substances other than sulphur dioxide, derived from matrices or from utilized chemicals.”

Taylor et al. (1986)²⁷ further described some key aspects of free and bound sulfites, namely the fate of these compounds as a function of food processing conditions (page 29):

“Another important factor in determining the fate of sulfites in foods is the nature of the processing treatments...sulfite levels can be altered in a number of ways: (1) The sulfites can be physically lost as SO₂ if the pH of the product drops below pH4.0, especially if the product is heated; (2) much of the sulfites in nonacid products can be converted into combined sulfite adducts, many of which remain to be characterized; (3) some of this combined sulfite will be in the form of extremely stable products, which cannot be recovered by conventional methods, so it will be “lost” as far as analysis is concerned; and (4) oxidation of sulfite to sulfate can occur in some foods...” [p. 29]

²⁷ Taylor, S.L., Higley, N.A., and Bush, R.K. 1986. Sulfites in foods: uses, analytical methods, residues, fate, exposure assessment, metabolism, toxicity and hypersensitivity. *Adv. Food Res.*30: 1-76.

“Molecular Sulfur Dioxide” Cannot be Measured with Current Methods of Chemical Analysis

There have been decades of effort undertaken to try to optimize analytical methods development to more accurately measure SO₂ and its related species in food ingredients and in whole foods and beverages. But it is important to recognize at the outset that no method developed to date has been successful in accurately speciating the various chemical forms in foods, including SO₂ gas.

Monier-Williams Method

The standard method of analysis of S(IV) species in food, based on that devised by Monier-Williams²⁸ in 1927, and sometimes referred to as the “classical” Monier-Williams Method (and later revised as the “modified” Monier-Williams Method), involves prolonged boiling/distillation (up to 1.75 hours) of the sulfited food sample in a strongly acidic solution to release SO₂ gas from all S(IV) species present in the food. The volatilized SO₂ gas is next removed by distillation through a reflux condenser and then the SO₂ gas that comes over is trapped in a hydrogen peroxide solution, where it is oxidized to sulfuric acid, which is determined either by titration with alkali or gravimetrically. Researchers who have used this method for sulfite analysis have usually all agreed that this method has numerous constraints and is extremely time-consuming and laborious. In addition, it does not truly measure “total SO₂” since some forms of combined sulfites are not dissociated during the acidic distillation procedure. Also, this procedure can be subject to interference by other sulfur compounds in foods.

Under these conditions, the very low pH and the high temperature assist in the decomposition of any hydroxysulfonates present in the sample and allow all the S(IV) to be desorbed from solution as gaseous SO₂. It is well known that the S(IV)-content of most sulfited foods decreases with time, particularly when the foods are exposed to air repeatedly and undergo off-gassing. Such decreases with time are affected by temperature, pH and other processing conditions as well as by subsequent storage and food preparation in the home or in retail food establishments. It is also well recognized

²⁸ Monier-Williams, G.W. 1927. Determination of sulfur dioxide. *Analyst* (London) 52: 415-416.

that the results determined by the Monier-Williams method are subject to uncertainties at the 10 ppm detection level.

The Monier-Williams method is also known to be a less accurate method when a significant amount of volatile interfering compounds, particularly organic acids such as acetic acid, is present in the food. If volatile acids are present in food samples, the Monier-Williams technique will overestimate sulfite unless proper precautions are taken (see Kim et al., 1987²⁹ discussed below). In addition, the indirect photometric-HPLC method developed by Pizzoferrato et al.³⁰ (discussed above) is able to avoid the potential interference of volatile substances derived from food matrices.

“Optimized Monier-Williams” Method

Several modifications have subsequently been published to try to improve on the original Monier-Williams methodology. These modifications have been captured in the “Optimized Monier-Williams” method by research efforts led by the FDA, and this method is still found to be one of the most suitable methods for determining sulfites in foods.³¹ Briefly, in this optimized method, the sample is acidified and refluxed under a steady stream of scrubbed nitrogen. Sulfites are released as SO₂ gas, which is subsequently trapped in 3% hydrogen peroxide to form sulfuric acid by oxidation. The resulting sulfuric acid is then quantified by titration with NaOH, and the reportable lower level of detection is 10 ppm.

²⁹ Kim, H.-J., Park, G.Y. and Kim, Y.-K. 1987. Analysis of sulfites in foods by ion exclusion chromatography with electrochemical detection. *Food Technol.* 41: 85-91.

³⁰ Pizzoferrato, L., Di Lullo, G. and Quattrucci, E. 1998. Determination of free, bound and total sulphites in foods by indirect photometry-HPLC. *Food Chem.* 63: 275-279.

³¹ AOAC International. 2000. AOAC Official Method 990.28, Sulfites in Foods, Optimized Monier-Williams Method. *Official Methods of Analysis of AOAC International*, Arlington, VA, Chapter 47, pp. 29-30.

Daniels, D.H., Joe, F.L. Jr, Warner, C.R., Longfellow, S.D., Fazio, T. and Diachenko, G.W. 1992. Survey of sulphites determined in a variety of foods by the optimized Monier-Williams method. *Food Add. Contam.* 9: 283-289.

Hillery, B.R., Elkins, E.R., Warner, C.R., Daniels, D., and Fazio, T. 1989. Optimized Monier-Williams method for determination of sulfites in foods: Collaborative study. *J. Assoc. Off. Anal. Chem.* 12: 470-415.

Daniels et al.³² pointed out that there was considerable variability in sulfite levels found within lots of the same brand as well as between different brands of foods. They noted several factors that could have contributed to the variability in the sulfite levels, including: (1) off-gassing of SO₂ from the food after treatment with sulfiting agents; (2) differences in the initial treatment levels; (3) sulfite losses due to reactions with food components or oxidizing agents after sulfite treatment but before analysis; and (4) difficulty in preparing a homogeneous test sample. Because of these considerations, the analyses were performed as quickly as possible to minimize loss of sulfite due to volatilization or reactions with other food components.

Ion Chromatographic Methods

Kim³³ published the results of a large collaborative study using an alternative method, one employing ion exclusion chromatographic separation and electrochemical detection (IEC-EC). At that time the official method for sulfite analysis was still the Monier-Williams method described above, and newer methods came under two categories: (1) acid distillation is used just as in the Monier-Williams method to separate sulfite from the food matrix, but the alkali titration step is replaced by a more selective determinative step. The SO₂ gas collected in the trap is either separated by ion exchange chromatography and detected by conductivity measurements or determined by more selective detection techniques, such as redox titration, coulometric titration or polarography without further separation; and (2) a direct alkali extraction method was extensively investigated in an effort to eliminate the acid distillation step, and it was found that the reversibly bound sulfite was released more efficiently from the foods by alkali treatment than by acid digestion. When conditions are optimized to more completely release the bound sulfite at alkaline pH levels, it was recognized that the dissociation of the bound sulfite was more favored at higher pH on both equilibrium constant and rate considerations.³⁴

³² Daniels, D.H., Joe, F.L. Jr, Warner, C.R., Longfellow, S.D., Fazio, T. and Diachenko, G.W. 1992. Survey of sulphites determined in a variety of foods by the optimized Monier-Williams method. *Food Add. Contam.* 9: 283-289.

³³ Kim, H.-J. 1990. Determination of sulfite in foods and beverages by ion exclusion chromatography with electrochemical detection: Collaborative study. *J. Assoc. Off. Anal. Chem.* 73: 216-222.

³⁴ Wedzicha, B.L. 1992. Chemistry of sulphiting agents in food. *Food Add. Contam.* 9: 449-459.

Nevertheless, separation of sulfite from the alkali extract has also been a challenging problem. Fortunately, several novel separation techniques facilitated the selective determination of sulfite in the alkali extract of the foods. Examples include flow injection analysis, ion exclusion chromatography with electrochemical detection, headspace techniques and a reverse-phase ion pairing liquid chromatographic (LC) method with spectrophotometric detection.

There are well-known sources of error with all of these measurement techniques. Since sulfite reacts with various components of the foods both reversibly and irreversibly, accurate determination of total sulfite is a difficult task. The concentration of the extracted sulfite in the alkali buffer tends to decrease gradually due to oxidation and recombination with food constituents, but the oxidative loss can be minimized with the addition of mannitol. When the food is homogenized, certain chemical reactions can take place to produce compounds that are reactive toward sulfite, with the enzymatic browning reaction being a good example. Kim noted that the alkali extraction used in the IEC-EC method did not effectively release sulfite bound to certain pigments, such as the nonenzymatic browning reaction products.³⁵ Therefore, lower results could be obtained by the IEC-EC method than the Monier-Williams method. In addition, the IEC-EC method did not detect naturally occurring sulfite in *Allium* and *Brassica* vegetables according to the results of an earlier study by Kim³⁶.

Warner et al. (1990)³⁷, an FDA research group, developed a method to measure and differentiate between “free” and “reversibly bound” sulfite in foods that took advantage of sulfite’s well known ability to react with formaldehyde in foods to form the bisulfite addition product hydroxymethylsulfonate (HMS). These researchers developed an ion-pairing high-performance liquid chromatography method. While the methodologic

³⁵ Kim, H.-J. 1990. Determination of sulfite in foods and beverages by ion exclusion chromatography with electrochemical detection: Collaborative study. *J. Assoc. Off. Anal. Chem.* 73: 216-222.

³⁶ Kim, H.-J. 1989. Comparison of the ion exclusion chromatographic method with the Monier-Williams method for determination of total sulfite in foods. *J. Assoc. Off. Anal. Chem.* 72: 266-272.

³⁷ Warner, C.R., Daniels, D.H., Fitzgerald, M.C., Joe, F.L. Jr. and Diachenko, G.W. 1990. Determination of free and reversibly bound sulphite in foods by reverse-phase, ion-pairing high-performance liquid chromatography. *Food Add. Contam.* 7: 575-581.

details are not important to describe here, they found that the rate of dissociation of the reversibly bound sulfite was relatively slow at pH 3 but very rapid at pH 7, and they were able to exploit this difference in kinetics to develop a procedure to determine free and reversibly bound sulfite in a variety of foods. Thus, the inherent pH of a food can be used to try to distinguish free from bound forms of sulfite in foods. This work followed on from an earlier FDA study³⁸ that noted that the carbonyl-sulfite adducts showed maximum stability at pH 2 and that dissociation was favored at pH > 6.

Measurement of total sulfite as the summation of “free” sulfite and “reversibly bound” sulfite requires the release of the reversibly bound sulfite either by refluxing in strong acid (the Monier-Williams method) or by raising the pH with NaOH. Several studies have recommended that the pH of food samples be increased to between 9 and 12 to ensure complete release of bound sulfite. Furthermore, Kim et al.³⁹ pointed out that the important step in differentiating free and bound sulfite rests on acid treatment without heat to just measure free sulfite, and it is important to recognize that total sulfite will always be greater than the free sulfite, indicating the presence of a significant amount of reversibly bound sulfite.

Additional, more recent methodological improvements were summarized by Chung et al.⁴⁰, including differential pulse polarography, flow injection analysis, capillary electrophoresis and their own new method employing HPLC with fluorometric detection, but none of these newer methods is capable of measuring molecular SO₂ in foods.

In sum, what has been termed “free sulfite” in OEHHA’s documents is what we can measure when we test foods at acidic conditions and do not use heat in the analytical methods, but it is critical to understand that this is still not a measure of molecular SO₂; and what has been termed “bound sulfite” can only be measured analytically by taking

³⁸ Kim, H.-J., Park, G.Y. and Kim, Y.-K. 1987. Analysis of sulfites in foods by ion exclusion chromatography with electrochemical detection. *Food Technol.* 41: 85-91.

³⁹ Id.

⁴⁰ Chung, S.W.C., Chan, B.P.T. and Chan, A.C.M. 2008. Determination of free and reversibly-bound sulfite in selected foods by high-performance liquid chromatography with fluorometric detection. *J. Assoc. Off. Anal. Chem.* 91: 98-102.

the food sample to a high pH in the presence of heat, which breaks down an array of sulfite species that are eventually detected analytically as molecular SO₂, but again this is still not a measure of molecular SO₂ in the foods per se. During analytical procedures where the food is either acidified or made basic, sulfites and sulfite precursors are produced via the decomposition of the “bound sulfites,” and these resulting substances should be considered more accurately as simple “artifacts” produced by the analytical methodologies and not as the quantitative amount of molecular SO₂ (gas) that existed in the food.

OEHHA Should Exclude Sulfurous Acid (H₂SO₃) and Hydrated SO₂ (SO₂ •H₂O) from Chemical Species Subject to Warning Requirements

We concur with OEHHA’s conclusion (as stated in the ISOR) that any “...sulfites, bisulfites or metabisulfites...are not currently listed under Proposition 65 and that exposure to them, at any level, is not subject to the warning and discharge requirements of Proposition 65.” And based on our scientific evaluations and conclusions stated in our comments, we believe that OEHHA should also conclude that two additional chemical species, Sulfurous Acid (H₂SO₃) and Hydrated SO₂ (SO₂ •H₂O), should be excluded from being subject to Proposition 65 warning and discharge requirements, since neither of these chemical species existing in sulfite-containing foods is a listed substance.

OEHHA Should Insert “Gas” or “Which Exists as a Gas” More Often Where “Molecular Sulfur Dioxide” is Already Stated in Documents

In OEHHA’s Draft Proposal on the MADL (including in the Initial Statement of Reasons) and in the Interpretive Guideline for Dried Fruits, we believe that the texts could be more specific in many places where the term “molecular sulfur dioxide” is stated. For purposes of scientific accuracy, clarity and consistency, we urge OEHHA to insert the word “gas” where “sulfur dioxide” is stated and/or modify the term “sulfur dioxide” with the phrase “which exists as a gas.”

In summary, the comments submitted herein identify minor modifications to OEHHA's proposed calculations of the MADL that would result in a MADL for SO₂ of 2200 micrograms/day or greater. Each of these minor revisions to the proposed MADL would produce a MADL which is (1) compliant with the Proposition 65 regulations, (2) scientifically more appropriate than using default assumptions as proposed, and (3) appropriately conservative. These comments also provide a detailed review of food chemistry and SO₂. The listed chemical, sulfur dioxide, does not exist in foods and beverages and is thus not subject to Proposition 65 requirements.

GMA thanks OEHHA for taking these comments into consideration. If you have any questions or comments, please feel free to contact Maia Jack, Director- Science Policy, by phone at 202-639-5922 or email at MJack@gmaonline.org. We look forward to working together with OEHHA on this important issue.

Sincerely,



Leon H. Bruner, DVM, Ph.D.
Senior Vice President, Scientific and Regulatory Affairs
and Chief Science Officer

cc: George Alexeeff, Ph.D. – OEHHA Director
Carol Monahan-Cummins – OEHHA Chief Counsel
Lauren Zeise, Ph.D. – OEHHA Reproductive and Cancer Hazard Assessment Branch Chief
Jim Donald, Ph.D. – OEHHA Reproductive Toxicology and Epidemiology Section Chief
Jay Murray, Ph.D. - Murray & Associates
James Coughlin, Ph.D. - Coughlin & Associates

Appendix

Re-evaluation of the fetal body weight data in the Murray et al. 1979 study

Kenneth T. Bogen, DrPH DABT — Exponent, Inc. — August 20, 2012

Raw study data corresponding to the publication of Murray et al. (1979) were obtained from Dow Chemical Co. via the lead study author, Dr. Jay Murray. The raw study data obtained consisted of litter-specific fetal body weights for litters from groups of CF-1 mouse dams exposed to either 0 or 25 ppm SO₂, for 7 hours/day from days 6 through 15 of gestation. All of the individual litter data worksheets were signed and dated. The study data were transcribed from hand-written raw study data sheets into an Excel spreadsheet. The resulting Excel data table was then re-checked against the information written in the raw data sheets. A statistical re-analysis of the study data is summarized in Table 1. Briefly, the re-analysis compared the sets of litter-specific mean values of fetal body weight, by exposure group. Each set of litter-specific mean fetal-body-weight values is approximately normally distributed ($p < 0.05$) by Shapiro-Wilk tests (Royston 1992). The sets have approximately equal variance ($p = 0.18$) by F-test, and the sample mean values of these sets do not differ significantly (2-tail $p = 0.17$, 1-tail $p = 0.085$) by t-test (Kendal and Stuart 1979). Documentation of these calculations is attached.

Table 1. Fetal body weight data from the Murray et al. (1979) study and re-analysis

Parameter	Murray et al. (1979), Table 2		Reanalysis	
	0	25	0	25
Concentration of SO ₂ , ppm	0	25	0	25
No. litters	26	20 ^a	26	20
Fetal body weight, g (Mean ± 1 SD ^B)	1.05±0.11	1.00±0.08 ^c	1.051±0.114	1.009±0.084 ^d

^a Table 2 of Murray et al. (1979) reported 21 litters, which included one completely resorbed litter. This completely resorbed litter was not included in the original analysis or the reanalysis of fetal body weights.

^b Mean = mean of litter-specific body weights; SD = standard deviation of litter-specific mean body weights.

^c Reported as significantly lower than control fetal body weight ($p < 0.05$, by ANOVA).

^d Not significantly different from the mean of the control litter-specific mean fetal body weights (2-tail $p = 0.17$, 1-tail $p = 0.085$, by t-test).

References

Kendall M, Stuart A. 1979. The Advanced Theory of Statistics, Vol. 2: Inference and Relationship. 4th ed. MacMillan Publishing Co., NY, p. 159-160.

Murray FJ, Schwetz BA, Crawford AA, Henck JW, Quast JF, Staples RE.
Embryotoxicity of inhaled sulfur dioxide and carbon monoxide in mice and rabbits.
J Environ Sci Health C 1979; 13(3):233–250.

Royston P. Approximating the Shapiro-Wilk W-test for non-normality. Statist Computing 1992; 2(3):117–119.