FDA Foods Program Compendium of Analytical Laboratory Methods: Chemical Analytical Manual (CAM)

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PROGRAM AREA: Food Additives

METHOD TITLE: Determination of Sulfites in Food using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

VALIDATION STATUS: Single-laboratory validation and Multi-laboratory validation per the Guidelines for the Validation of Chemical Methods for the FDA Foods Program 3rd Edition

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METHOD SUMMARY/SCOPE:

> **Analyte(s):** Sulfites (all sulfites converted to hydroxymethylsulfonate through the extraction process and then converted to sulfur dioxide during quantitation).
>
> **Matrices:** Dried fruits and vegetables, shrimp, juices and sweeteners, *Allium* containing products, *Brassica* vegetables, canned fruits and vegetable, and spices.

The test sample is mixed with a 0.2% formaldehyde extraction solution to convert the free sulfite to a more stable adduct, hydroxymethylsulfonate (HMS). The HMS is extracted by rotating, sonicating, and centrifuging the test sample. A C18 solid phase extraction (SPE) cartridge is used to clean up the extract and the eluent is heated at 80°C for 30 minutes to convert any sulfite-carbonyl adducts to HMS. The cooled extract is combined with internal standard in LC vials and analyzed using LC-MS/MS. HMS ions are identified by retention time and ion ratio matching with the calibration standards. Quantitation is performed using an external calibration curve prepared in solvent. The concentration of sodium sulfite is determined using the peak area ratio of response of the HMS quantitation transition to that of the [^{34}S]-IS, and calculating the concentration by preparing a calibration curve using the ratio for the calibration standard to that of the [^{34}S]-IS. After determination of the concentration from the curve, the concentration must be adjusted for dilution and starting sample mass prior to converting from Na_2SO_3 to SO_2 using the molar ratio of 126 to 64.

REVISION HISTORY: Version 004.04 replaces version 004.03 (2023). Based on discussions in the Chemical Research Coordinating Group, additional validation information was added to the method to assist in method implementation. Additional information was also added regarding quality control criteria and validation data. In addition, the scope table was updated to include additional matrices that have since been validated.

Version 004.03 replaces version 004.02 (2021). Based on discussions in the Food Additive Operations Committee some clarifications were made with the method. In addition, the scope table was updated to include additional matrices that have since been validated.

Version 004.02 replaces version 004.01 (2020). In 2020, the method was revised to include an additional extraction technique for high moisture solids such as jarred peppers or canned tuna. Additional validation data for *Allium* (ex. garlic) and *Allium*-containing products was included.

OTHER NOTES:

Determination of Sulfites in Food using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

Version 2023 (2023)

Author: Katie Carlos

CFSAN/ORS reviewers: Lowri de Jager, Tim Begley

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2023.1 METHOD TITLE: Determination of Sulfites in Food using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

2023.2 SCOPE OF APPLICATION

The method describes a procedure for measuring free sulfite plus a reproducible portion of bound sulfite in food using LC-MS/MS. Sulfiting agents and their synonyms may include sulfur dioxide, sodium sulfite, sodium bisulfite, potassium bisulfite, sodium metabisulfite, and potassium metabisulfite. The method has been validated in the following food matrices:

Matrix	Validation	Date	Analyst
Dried apricot, Dried pineapple, Dried coconut, Crystallized ginger, Dried potato, Dried vegetable mix, Bamboo shoots, White grape juice, Red wine vinegar, Molasses, Apricot jam, Shrimp	Single lab validation[1]	2014	Katie Carlos (Robbins)
Dried apricot, Dried vegetable mix, White grape juice, Shrimp	Multi-lab validation[2]	2016	POC: Katie Carlos 11 laboratories participated

Basic Modification

Matrix	Validation	Date	Analyst
Allium genera included on ingredient list (not pure *Allium*): hummus, quinoa, baked phyllo shells, and potato chips	Single lab matrix extension[3]	2020	POC: Katie Carlos
Sugar Palm Fruit (Kaong)	Single lab verification	2021	POC: DENLHAF
Chutney	Single lab verification	2021	POC: NYLHAF
Gummy Bears	Single lab verification	2021	POC: NYLHAF
Preserved Radish	Single lab verification	2021	POC: DENLHAF
Red Melon Seeds	Single lab verification	2022	POC: NYLHAF
Mini muffins	Single lab verification	2022	POC: NYLHAF
Chocolate Cereal	Single lab verification	2022	POC: ATLHAF
Diced white potatoes (canned in water)	Single lab verification	2022	POC: DENLHAF
Soybean Paste	Single lab verification	2022	POC: DENLHAF
Flour tortillas	Single lab verification	2022	POC: DENLHAF

Low Moisture Solids

Matrix	Validation	Date	Analyst
Brassica genera: cabbage, broccoli, cauliflower, kale, Brussels sprouts	Single lab investigation[4]	2016	POC: Katie Carlos
Pure *Allium* genera	Single lab investigation[3,4]	2016 and 2020	POC: Katie Carlos
Dried Banana Chips	Single lab verification	2021	POC: DENLHAF
Sundried Tomatoes	Single lab verification	2021	POC: DENLHAF
Masala Powder	Single lab verification	2021	POC: NYLHAF
Saltine Crackers	Single lab verification	2022	POC: NYLHAF
Coconut Cookies	Single lab verification	2022	POC: NYLHAF

High Moisture Solids

Matrix	Validation	Date	Analyst
High moisture matrices: canned peaches, canned apricots, canned tuna (in both oil and water), jarred pickles.	Single lab validation	2019	POC: Katie Carlos

Coconut/High Fat Modification

Matrix	Validation	Date	Analyst
Pecans	Single lab verification	2021	POC: NYLHAF
Sesame Brittle	Single lab verification	2021	POC: NYLHAF
Peanut Brittle	Single lab verification	2022	POC: NYLHAF
Smoked Salmon	Single lab verification	2022	POC: NYLHAF
Halawa (Tahini paste)	Single lab verification	2022	POC: NYLHAF

Liquid Modification

Matrix	Validation	Date	Analyst
Energy Drink	Single lab verification	2022	POC: ATLHAF

This method should be used by analysts experienced in the use of LC-MS/MS, including but not limited to operation of the instrumentation and software, data analysis and reporting results. Analysts should also be able to identify chromatographic and mass spectrometric interferences during sample analysis and take necessary actions following validated procedures for their correction to achieve reliable

identification and quantitation. The method should be used only by personnel thoroughly trained in the handling and analysis of samples for the determination of food additives in food and beverage products.

The original method did not apply to members of the *Allium* (garlic, onion, leeks, chives, etc.). Subsequent research has provided more information to better understand the degree to which a false positive response is present in *Allium* and *Allium* containing products. Due to this research, the method can now be used for the analysis of *Allium* containing products. For pure Allium samples, the false positive response ranged from 25-98 ppm SO_2 for eight commercial garlic powders investigated. With background subtraction, added sulfite could be adequately recovered from both fresh and roasted garlic. Fresh garlic had recoveries of 125 ± 1% and roasted garlic had recoveries of 116 ± 1% for a 10 ppm SO_2 spike.

The original multi-laboratory validation did not include the *Brassica* genera (cabbage, broccoli, cauliflower, kale, etc.). However, a thorough investigation of the blank concentration in five different Brassica vegetables (cabbage, broccoli, cauliflower, kale, Brussels sprouts) was conducted.[4] All of the vegetables had SO_2 concentrations below 5 ppm. Due to these data, method extension to include the Brassica genera was supported. These samples can be prepared following the dried sample preparation. The publication should be consulted prior to running the method on *Brassica* vegetables.

The original sulfite method, which successfully passed a multi-laboratory validation in 2016, covered modifications based on moisture and fat. There was a basic method plus three additional modifications included in the method development and validation. The basic method was developed mainly for dried fruit, the liquid modification for juices and sweeteners, low moisture modification for dried vegetables, and high fat modification for coconut. After the validation was complete, it became apparent that an additional modification would be necessary for high moisture solids such as canned tuna, pickles in brine, and canned fruit. This modification was validated using a Level 2 single laboratory validation (SLV): five different matrices were spiked at three concentrations.

2023.3 PRINCIPLE

The test sample is mixed with a 0.2% formaldehyde extraction solution to convert the free sulfite to a more stable adduct, hydroxymethylsulfonate (HMS). The HMS is extracted by rotating, sonicating, and centrifuging the test sample. A C_{18} SPE cartridge is used to clean up the extract and the eluent is heated at 80 °C for 30 minutes to convert any sulfite-carbonyl adducts to HMS. The cooled extract is combined with internal standard, $Na_2{}^{34}SO_3$, in LC vials and analyzed using LC-MS/MS. The HMS ions are identified by retention time and ion ratio matching with the calibration standards. Quantitation is performed using an external calibration curve prepared in solvent. The concentration of sodium sulfite is determined using the peak area ratio of response of the HMS quantitation transition to that of the [^{34}S]-IS, and calculating the concentration by preparing a calibration curve using the ratios for the calibration standard to that of the [^{34}S]-IS. After determination of the concentration from the curve, the concentration must be adjusted for dilution and starting sample mass prior to converting from Na_2SO_3 to SO_2 using the molar ratio of 126 to 64.

2023.4 REAGENTS

The use of trade names in this method constitutes neither endorsement nor recommendation by the U. S. Food and Drug Administration (FDA). Equivalent performance may be achievable using apparatus and materials other than those cited here. During the multi-laboratory validation (MLV), laboratories used reagents of equivalent specifications and achieved accepted results.

(1) Formaldehyde – 37% aqueous solution, ACS reagent, stabilized with 10-15% methanol (Sigma-Aldrich).
(2) Ammonium acetate – SigmaUltra, minimum 98%, LC-MS grade recommended (Sigma-Aldrich).
(3) Water- 18 MΩ water
(4) Acetic acid – Ultrex glacial (J. T. Baker).
(5) Optima LC-MS grade water (Fisher Scientific)
(6) Optima LC-MS grade acetonitrile (Fisher Scientific)
(7) Methylene chloride (Fisher Scientific)
(8) Methanol (Fisher Scientific)

2023.5 STANDARDS

(1) Sodium sulfite (Na_2SO_3). – 98% ACS reagent (Sigma-Aldrich)
(2) Sodium sulfite stable isotope ($Na_2{}^{34}SO_3$). – 95% (Sigma-Aldrich)

2023.6 PREPARATION OF SAMPLES OR TEST PORTIONS

2023.6.1 Prepare 2.0% Formaldehyde Extraction Solution

(1) Accurately weigh 1.925 g ammonium acetate.
(2) Quantitatively transfer the ammonium acetate to a 500 mL volumetric flask using a small amount of 18 MΩ water.
(3) Add approximately 100 mL of 18 MΩ water to the flask to fully dissolve the ammonium acetate.
(4) Using a 50 mL graduated cylinder, transfer 27 mL of formaldehyde to the volumetric flask.
(5) Dilute with 18 MΩ water to 500 mL, cap the volumetric flask and invert several times to ensure proper mixing.
(6) Properly calibrate the pH meter according to the manufacturer's recommended directions.
(7) Place all of the solution (500 mL) in a 600 mL beaker with a stir bar. Measure the pH of the solution. The pH of this solution should be between 4 and 5.
(8) Adjust the pH using dropwise addition of acetic acid to a final pH of 4.5.
(9) The 2.0% formaldehyde solution can be stored for up to 3 months in a sealed glass container at room temperature. This solution can be used to make standard solutions stored past this expiration date if they are prepared before the expiration date of the formaldehyde solution and then stored at refrigeration or freezer temperatures (ex. 4 °C or -20 °C). The rationale behind this is that the formaldehyde itself can degrade over time but once the sulfite solutions are

prepared a very stable adduct, HMS, is formed that will not degrade on the same timeline.

2023.6.2 Prepare 0.2% Formaldehyde Extraction Solution

(1) Using the graduated cylinder, transfer 100 mL of the 2.0% formaldehyde extraction solution to a 1000 mL volumetric flask.
(2) Dilute to volume with the 18 MΩ water. Place the glass stopper in the flask and invert to mix.
(3) This solution can be stored for up to two (2) weeks in a sealed glass container at room temperature.

2023.6.3 Prepare 10 mg/mL Na_2SO_3 Standard Stock Solution

Note: Prepare two independent standards for continuing calibration verification (CCV) and initial calibration verification (ICV).

(1) Using an analytical balance, weigh out 100 ± 3.0 mg of sodium sulfite.
(2) Record the mass so that the exact concentration can be determined later using the mass and purity of the sodium sulfite.
(3) Quantitatively transfer the sodium sulfite to the 10 mL flask using the 2.0% formaldehyde solution.
(4) Dilute to volume with the 2.0% formaldehyde solution, sonicating if necessary to ensure that the sulfite is completely dissolved.
(5) Transfer to 20 mL amber glass vial and store at 4 °C. This solution can be stored for up to one (1) year at 4 °C when the solution is made using the 2.0% formaldehyde diluent within its 3-month expiration. As mentioned above, the 2% formaldehyde degrades over time, but the standards form a stable formaldehyde adduct, hydroxymethylsulfonate (HMS). Therefore, the standards are stable past the expiration date of the formaldehyde.

2023.6.4 Prepare 25 ppm $Na_2{}^{34}SO_3$ Internal Standard Stock Solution

(1) Using an analytical balance, weigh out 2.5 ± 0.05 mg of sodium sulfite.
(2) Record the mass so that the exact concentration can be determined later using the mass and purity of the sodium sulfite.
(3) Quantitatively transfer the sodium sulfite to the flask using the 2.0% formaldehyde solution.
(4) Dilute to volume with the 2.0% formaldehyde solution, sonicating if necessary to ensure that the sulfite is completely dissolved.
(5) Transfer to the 100 mL glass bottle and store at 4 °C. This solution can be stored at 4 °C for 1 year and can be stored at -20 °C for up to 3 years. Laboratories can evaluate extending expiration dates if desired but the response of the internal standard must compare within 50% to the initial response observed with the fresh standard.

2023.6.5 Prepare 5 ppm $Na_2{}^{34}SO_3$ Internal Standard Stock Solution

(1) Dilute the 25 ug/mL $Na_2{}^{34}SO_3$ standard 1:5 by pipetting 2 mL of the standard into an empty 10 mL volumetric flask. Dilute to volume using 0.2% formaldehyde solution and cap and invert to mix. This is the 5 ppm $Na_2{}^{34}SO_3$ standard.

(2) Transfer to the 20 mL vial and store at 4 °C. The solution expiration is 3 months from preparation date (when using the 0.2% formaldehyde diluent within its 2-week expiration).

2023.6.6 Prepare 1 M Ammonium Acetate Solution

(1) Using an analytical balance, weigh out 7.7 ± 0.05 g of ammonium acetate.

(2) Quantitatively transfer the ammonium acetate to the flask using the LC-MS grade water. If needed, a funnel may be used to help with the transfer.

(3) Dilute to volume with the water and invert several times to mix.

(4) Transfer to a glass storage bottle and store at room temperature.

2023.6.7 Prepare Mobile Phase A (90% ACN in 10 mM ammonium acetate).

(1) Combine 10 mL of 1 M ammonium acetate, 90 mL of LC-MS water and 900 mL of ACN in a 1 L mobile phase bottle. Cap and thoroughly mix.

2023.6.8 Prepare Mobile Phase B (50% ACN in 10 mM ammonium acetate).

(1) Combine 10 mL of 1 M ammonium acetate solution, 490 mL of LC-MS water and 500 mL of ACN in a 1 L mobile phase bottle. Cap and mix thoroughly.

2023.6.9 Sample Preparation

General: Refer to the compliance program for sample compositing. Samples should be stored frozen until analysis unless analyzed within 24 hours. On the day of analysis, samples should be removed from the freezer and allowed to thaw. Samples are extracted using 0.2% formaldehyde solution which converts all free sulfite to a more stable adduct, hydroxymethylsulfonate (HMS). Two extractions are conducted to ensure that all of the free and bound sulfite has been extracted from the food matrix. A sonication step is included to increase extraction yield.

For sulfite samples analyzed with the LC-MS/MS method, no check analysis is required. The samples should be prepared and analyzed in duplicate. A percent relative difference should be calculated for the two extracts and the percent difference should be less than 20%.

NOTE: IF SAMPLE IS A LIQUID, HIGH-MOISTURE SOLID, DRIED VEGETABLE OR COCONUT THEN THERE ARE MODIFICATIONS TO THIS PREPARATION INCLUDED IN SECTIONS 6.10-6.12.

(1) Place a 50 ± 0.5 g portion of the sample into a variable speed laboratory blender.

(2) Tare a blank beaker and accurately weigh 100 ± 3 g of 0.2% extracting solvent. Record mass added. Add to the laboratory blender.

(3) Homogenize the sample using the laboratory blender until thoroughly blended. This should take ~2 minutes.
(4) Accurately weigh 15.0 ± 0.5 g of homogenate into 50 mL centrifuge tube; record mass added.
(5) Add 20 mL of 0.2 % formaldehyde solution to centrifuge tube and place on a tube rotator at 70 rpm for 10 min.
(6) Transfer tubes to a beaker and sonicate for 8 minutes.
(7) Remove tubes from the sonicator and centrifuge at 4000 rcf for 5 min. Note: Some particularly light samples such as dried vegetables will still float on the surface under these centrifugation conditions. Higher speeds than 4000 rcf can be used in these cases in an effort to pack the sample better.
(8) Decant supernate into 50 mL stoppered graduated cylinder.
(9) Add an additional 20 mL of 0.2% formaldehyde solution to original falcon tube and repeat extraction steps (5-7). Before placing tubes on the rotator, use a vortex to ensure that everything has been properly mixed.
(10) Add supernate to stoppered graduated cylinder and dilute to 50 mL with 0.2% formaldehyde solution. Place the glass stopper and invert to mix.

2023.6.10 Modifications for Products with High Moisture Contents – Liquids (Vinegars, Juices and Syrups)

(1) Place 1.00 ± 0.05 g of sample into a 10 mL volumetric flask using the analytical balance. Dilute to volume using 0.2% formaldehyde solution. Record the mass of the sample. Invert to mix.
(2) Proceed with the SPE clean-up and heating derivatization as described in step 2023.6.15.

2023.6.11 Modifications for Products with High Moisture Contents – Solids (Canned Fruit, Canned Seafood, Jarred Pickles and Peppers)

(1) If not already completed, create a homogenate of the composite leaving a slurry that can be analyzed as one without chunks.
(2) Place 50.0 ± 0.5 g of slurried composite into a variable speed blender.
(3) Tare a blank beaker and accurately weigh 100 ± 3 g of 0.2% extracting solvent. Record mass added. Add to the laboratory blender.
(4) Homogenize the sample using the laboratory blender until thoroughly blended. This should take ~2 minutes.
(5) Accurately weigh 15.0 ± 0.5 g of homogenate into 50 mL centrifuge tube; record mass added.
(6) Add 15 mL of 0.2 % formaldehyde solution to centrifuge tube and place on a tube rotator at 70 rpm for 10 min.
(7) Transfer tubes to a beaker and sonicate for 8 minutes.
(8) Remove tubes from the sonicator and centrifuge at 4000 rcf for 5 min.
(9) Decant supernate into 50 mL stoppered graduated cylinder.
(10) Add an additional 20 mL of 0.2% formaldehyde solution to original falcon tube and repeat extraction steps (6-8). Before placing tubes on the rotator, use a vortex to ensure that everything has been properly mixed.

(11) Add supernate to stoppered graduated cylinder and dilute to 50 mL with 0.2% formaldehyde solution. Place the glass stopper and invert to mix.

2023.6.12 Modifications for Products with Low-Moisture Contents (Dried vegetables, dried bananas, cereals, crackers, baked goods, dried soup mix)

(1) Optional step: If the sample is not a fine powder, grind the entire sample using a blender. Weigh out 5.00 ± 0.25 g of dried vegetable directly into a 50 mL centrifuge tube.
(2) Add 30 mL of 0.2 % formaldehyde solution to centrifuge tube.
(3) Place on a tube rotator at 70 rpm for 10 min. Transfer tubes to a beaker and sonicate for 8 minutes.
(4) Remove tubes from the sonicator and centrifuge at 4000 rcf for 5 min.
(5) Decant supernate into 50 mL stoppered graduated cylinder.
(6) Add an additional 20 mL of 0.2% formaldehyde solution to original falcon tube and repeat extraction steps (3-5).
(7) Add supernate to stoppered graduated cylinder and dilute to 50 mL with 0.2% formaldehyde solution.
(8) Continue with the SPE clean-up and heating derivatization as described in step 2023.6.15.
(9) Note: Use 0.45 µm filter (yellow) instead of 0.20 µm filter (blue) for low-moisture modification (See 2023.7).

2023.6.13 Modifications for Shredded Coconut

(1) Begin sample preparation using the same method as listed in step 6.9 through the first centrifuge step. At this point there should be three layers in the centrifuge tube: a bottom coconut layer, a middle layer of extracting solution, and a top layer of lipid. This top layer can make pouring off into the 50 mL cylinder difficult. Instead of pouring into the cylinder, filter through a frit and then add the liquid into the graduated cylinder.
(2) Place the 20 mL empty cartridge with added frit onto a vacuum manifold. Place a beaker in the manifold to catch the flow coming through the cartridge.
(3) Turn on the vacuum flow and add the sample to the cartridge. Set the vacuum so that a dropwise flow is observed. Try to keep the lipid layer from falling out of the centrifuge tube. Once the dropping has stopped, turn off the vacuum and remove the beaker. Transfer the extract to the 50 mL stoppered graduated cylinder.
(4) Add an additional 20 mL of 0.2% formaldehyde solution to original falcon tube and repeat extraction steps (rotator, sonicator, and centrifuge).
(5) Repeat filtering through the empty cartridge. Add additional extract to the same stoppered centrifuge tube rinsing with additional 0.2% formaldehyde solution.
(6) Continue with the SPE clean-up and heating derivatization as described in step 2017.6.15 but reduce the volume of extract used to 1.5 mL.

2023.6.14 Quality Control Samples

Quality Control samples are processed through the entire extraction concurrently with each set of samples. The selected control matrix should be a matrix-matched commodity similar to the matrix of the sample(s), as defined by the FDA Foods Program Guidelines for the Validation of Chemical Methods,[5] 3rd Edition, Table A4.1.

2023.6.14.1. Reagents

(1) 0.2% formaldehyde extraction solution (prepared in step 6.2)
(2) 10 mg/mL Na_2SO_3 standard (prepared in step 6.3)

2023.6.14.2. Equipment

(1) Blender or food processor – variable speed with 500 mL capacity
(2) Analytical balance
(3) Polypropylene tubes (centrifuge tubes), 50 mL or volumetric flask, 10 mL (depending on method protocol to be used)
(4) Representative matrix-match commodity

2023.6.14.3. Procedure

2023.6.14.3.1 Method Blank

Using 0.2% formaldehyde extracting solution (prepared in step 6.2)

(1) Based on the extraction protocol or modification in use, accurately weigh the appropriate amount of total extraction solvent into a 50 mL centrifuge tube or appropriate glassware.
(2) Proceed with extraction method as outlined in section 6.9-6.13.

2023.6.14.3.2 Matrix Blank

Using an appropriate matrix-matched commodity

(1) Based on the extraction protocol or modification in use, accurately weigh the appropriate amount of selected matrix-matched commodity into the appropriate container (i.e., blender jar, centrifuge tube, or volumetric flask).
(2) Add extraction solution as outlined per the method protocol or modification below (section 6.9-6.13).
(3) Proceed with method as outlined in sections 6.9-6.13.

2023.6.14.2.3 Matrix Spike and Matrix Spike Duplicate

Using the same selected matrix-match commodity as in 6.14.3.B (Matrix Blank).

(1) Based on the extraction protocol or modification in use, accurately weigh the appropriate amount of selected matrix-matched commodity into the appropriate container (i.e., blender jar, centrifuge tube, or volumetric flask).
(2) Spike sample at 10 µg/g (ppm) Na_2SO_3.
(3) Proceed with method as outlined in sections 6.9 to 6.13.

2023.6.14.2.4 Matrix Extensions

For a matrix type not listed in the scope table of the compendium method, use the following protocol to analyze the sample.

(1) Consult the FDA Foods Program Guidelines for the Validation of Chemical Methods, 3rd Edition,[5] Table A4.1 and CFSAN to analyze the sample using the most appropriate extraction protocol based on matrix type.

(2) If SO_2 is not found or found at or below the action level of 10 µg/g (ppm)
   a. Spike the sample in duplicate at 10 µg/g (ppm).
   b. Recovery should be within range of those reported for the original multi-laboratory validation (80%-120%).

(3) If SO_2 is found above the action level of 10 µg/g (ppm)
   a. Spike the sample in duplicate at an acceptable concentration (50-100 %) and calculate recovery with background subtraction.
   b. Recovery should be within the range of those reported for the original multi-laboratory validation (80%-120%).

(4) In situations where the new matrix does not meet method performance criteria (80-120%), the Monier-Williams (MW) method is recommended for quantitation and reporting.
   a. Validation of the new matrix, as a method extension, should be performed per the FDA Food Guidelines for the Validation of Chemical Methods, 3rd Edition,[5] when sample workload permits.
   b. These results would need to be reviewed by the Food Additive Technical Advisory Group (TAG) prior to use for regulatory samples.

(5) The results should be shared with the lab supervisor, the local QA manager, and the TAG. Once reviewed and approved, the matrix can now be analyzed by other regulatory labs using the harmonized method without further validation.

2023.6.15 SPE Clean-up and Heating Derivatization

General: Sample is applied to a C18 SPE cartridge to remove all lipophilic matrix components. The eluent is then heated at 80 ± 5 °C for 30 minutes to convert all sulfite-carbonyl adduct to the HMS adduct of interest.

(1) Condition a C18 SPE cartridge by rinsing sequentially with 3 mL portions of methylene chloride, methanol and 0.2 % formaldehyde solution. Vacuum pressure should be adjusted to allow for a drop-wise flow.

(2) Pass 2 mL of sample extract through the cartridge and discard.

(3) Pass 2 additional mL of sample extract through the cartridge and collect the eluent into a 4 mL screw-cap vial.

(4) Cap vial. Set the tube heater to 80 °C and allow to reach the setpoint. Heat vial in the tube heater for 30 min. Maintain the heater at 80 ± 5°C throughout the heating time.

(5) Remove vial from tube heater and cool to room temperature.

2023.7 APPARATUS/INSTRUMENTATION

The use of trade names in this method constitutes neither endorsement nor recommendation by the U. S. Food and Drug Administration (FDA). Equivalent performance may be achievable using apparatus and materials other than those cited here. Please refer to Appendix 6 of the Chemical Method Validation Guidelines[5] when changing materials to ensure you are staying within the constraints of the method definitions. Please refer to the 'Instrument Performance Criteria Evaluation' section (2023.11) for information on how to ensure instrument performance meets requirements for method analysis. During the MLV, laboratories used reagents of equivalent specifications and achieved accepted results. The instrumentation described here was included in the original method validation.

(1) pH meter
(2) Blender – Variable speed Waring laboratory blender with 500 mL glass jar
(3) Centrifuge – Marathon 21000R refrigerated centrifuge (Fisher Scientific)
(4) Sonicator- Branson 2510 ultrasonic cleaner (Sigma-Aldrich)
(5) SPE vacuum manifold
(6) Tube heater – Techne Sample Concentrator Dri-Block DB-3A (Bibbey Scientific US, Burlington, NJ)
(7) Solid phase extraction cartridges – Bakerbond C18, 6 mL, 500 mg (J. T. Baker Chemical Co., cat # 7020-26)
(8) Liquid chromatograph – Waters Acquity Ultra-performance LC system, consisting of a binary solvent manager and a sample manager.
(9) Mass spectrometer – Applied Biosystems 4000 Q-trap LC-MS/MS system with electrospray source in the negative ion mode using Analyst software.
(10) External switching valve – Valco Instruments Co., Houston, TX
(11) Analytical column – 150 x 2.1 mm SeQuant ZIC HILIC, 5 µm. (The Nest Group, Inc., Southborough, MA). Thermostat column to 30 °C.
(12) Syringe filters – 17 mm, 0.2 µm PTFE syringe filters (Titan brand, Fisher-Scientific)
(13) Syringe filters (for modification) – 17 mm, 0.45 µm PTFE syringe filters (Titan brand, Fisher-Scientific)

2023.8 METHOD

2023.8.1 Prepare Standard Curve for LC-MS/MS Analysis (THIS SHOULD BE DONE DAILY)

THE CALIBRATION CURVE MUST BE PREPARED WITHIN 24 HOURS OF THE SAMPLES INCLUDED IN THE BATCH. IF PROBLEMS DEVELOP THAT DELAY INSTRUMENT ANALYSIS, ALL PREPARED VIALS SHOULD BE STORED IN THE FRIDGE. IN THIS CASE, INSTRUMENTAL ANALYSIS MUST BE COMPLETED WITHIN 5 DAYS OF PREPARATION.

(1) Dilute the 10 mg/mL Na_2SO_3 standard 1:10 by pipetting 1 mL of the standard into an empty 10 mL volumetric flask. Dilute to volume using 18 MΩ ultrapure water and cap and invert to mix. This is the 1000 ppm Na_2SO_3 standard.

(2) Dilute the 1000 ppm Na_2SO_3 standard 1:10 by pipetting 1 mL of the standard into an empty 10 mL volumetric flask. Dilute to volume using 0.2% formaldehyde and cap and invert to mix. This is the 100 ppm Na_2SO_3 standard.

(3) Dilute the 100 ppm Na_2SO_3 standard 1:10 by pipetting 1 mL of the 100 ppm standard into an empty 10 mL volumetric flask. Dilute to volume using 0.2 % formaldehyde, cap and invert to mix. This is the 10 ppm Na_2SO_3 standard.

(4) Dilute the 10 ppm Na_2SO_3 standard 1:10 by pipetting 1 mL of the 10 ppm standard into an empty 10 mL volumetric flask. Dilute to volume using 0.2 % formaldehyde solution, cap and invert to mix. This is the 1 ppm Na_2SO_3 standard.

(5) Pipet into the 2 mL clear glass vials per the scheme shown in table below in the following order: Na_2SO_3, 0.2% formaldehyde solution, $Na_2{}^{34}SO_3$, and LC-MS grade ACN. Cap vials.

Concentration (µg/mL)	**Na_2SO_3 solution Concentration (ppm)**	**Na_2SO_3 solution Volume (µL)**	**0.2% extracting solution (µL)**	**$Na_2{}^{34}SO_3$ (µL)**	**ACN (µL)**	**Total (µL)**
0.01*	1	10	190	100	700	1000
0.02*	1	20	180	100	700	1000
0.05	1	50	150	100	700	1000
0.1	10	10	190	100	700	1000
0.2	10	20	180	100	700	1000
0.4	10	40	160	100	700	1000
0.8	10	80	120	100	700	1000
1.5	100	15	185	100	700	1000
3.0	100	30	170	100	700	1000
4.5	100	45	155	100	700	1000

*These concentrations were included in the validation studies but are not included for routine regulatory analyses.

2023.8.2 Prepare Samples for LC-MS/MS Analysis

(1) Pipet 200 µL of the cooled extract into the clear glass vial. Pipet 100 µL of the 5 ppm $Na_2{}^{34}SO_3$ standard into the same vial along with 700 µL of acetonitrile.

(2) Cap the vial and shake. A visible precipitate may have formed at this point.

(3) Pour the contents of the vial into a syringe with a 0.2 um syringe filter attached. If the sample is a dried vegetable or dried potato sample, use a 0.45 µm syringe filter instead. Filter syringe contents into a new clear glass vial and cap.

2023.8.3 LC-MS/MS Analysis

General: The MS/MS data were acquired using the MRM mode (unscheduled) of an AB Sciex 4000 QTRAP. An Acquity Ultraperformance LC System (Waters, Milford, MA) was used for method development and validation. An Agilent 1290 Infinity, Shimadzu LC-20, and Shimadzu LC-30 has also been used for sample analysis. Slight adjustments to dilution factor and declustering potential may be necessary if a different MS system is utilized.

2023.8.3.1 Method Parameters

NOTE: Author can be contacted for information on conditions for additional instrumentation not included here.

(1) Set up the LC-MS/MS method with the following parameters and monitor for the transitions using the information in the table below.

NOTE: Every MS system may optimize individual transitions slightly differently. Variances of several orders of magnitude have been observed between different instruments for these transitions. If this is the case and the laboratory finds that the instrument responses are significantly higher or lower than the magnitude range that would be expected, re-optimize the collision energy (CE) for that particular transition to make adjustments in the sensitivity. Authors can be contacted for assistance with this issue. Optimizations should maintain (or improve) the performance of the method.

2023.8.3.1.1 MS/MS Conditions for the Monitored Transitions on a 3500 or 4000 QTRAP

Q1 mass (*m/z*)	Q3 mass (*m/z*)	ID	Dwell Time	DP (V)	EP (V)	CE (V)	CXP (V)
111	81	HMS	170	-25	-4	-15	-6
111	80	HMS	550	-25	-4	-40	-6
113	83	HMS (^{34}S)	80	-25	-4	-15	-6
113	82	HMS (^{34}S)	80	-25	-4	-40	-6

2023.8.3.1.2 MS/MS Conditions for the Monitored Transitions on a 4500 or 5500 QTRAP

Q1 mass (*m/z*)	Q3 mass (*m/z*)	ID	Dwell Time	DP (V)	EP (V)	CE (V)	CXP (V)
111	81	HMS	170	-25	-4	-15	-8
111	80	HMS	550	-25	-4	-40	-6
113	83	HMS (^{34}S)	80	-25	-4	-15	-8
113	82	HMS (^{34}S)	80	-25	-4	-40	-6

2023.8.3.1.3 Gradient Profile for the LC Conditions

Time (min)	Concentration of B
0.0	10%
6.0	10%
10.0	50%
15.75	50%
16.00	10%
24.00	10%

2023.8.3.1.4 Time Profile for the External Switching Valve

Time (min)	Direction
0.0	To Waste
6.0	To MS
9.0	To Waste

2023.8.3.1.5 Method Parameters

Set up the LC-MS/MS method with the following parameters and monitor for the transitions using the information included above and below. Adjustments to parts 1-6 are allowed in order to optimize each instrument. No adjustments may be made to parts 7-9.

(1) The following conditions are for the 3500 triple quad:
   a. Curtain gas: 35 au
   b. Collisionally activated dissociation (CAD) gas: 8
   c. Ion spray voltage: -1100 V
   d. Source temperature: 550 °C
   e. Gas 1 pressure: 50 au
   f. Gas 2 pressure: 50 au
   g. Injection volume: 5 µL
   h. Column temperature: 30 °C
   i. Flow rate: 0.30 mL/min

(2) The following conditions are for the 4000, 4500, and 5500 Q-trap:
   a. Curtain gas: 35 au
   b. Collisionally activated dissociation (CAD) gas: medium
   c. Ion spray voltage: -1200 V
   d. Source temperature: 550 °C
   e. Gas 1 pressure: 70 au
   f. Gas 2 pressure: 40 au
   g. Injection volume: 5 µL
   h. Column temperature: 30 °C
   i. Flow rate: 0.30 mL/min

2023.8.3.2 Method Run Order Template

Run the samples using the following template:

(1) Blank (ACN)
(2) Standard Curve
(3) CCV
(4) ICV
(5) Blank (ACN)
(6) Method Blank (0.2% formaldehyde solution)
(7) Matrix Blank (matrix-match commodity)
(8) Matrix Spike (matrix-match commodity spiked at 10 ppm)

(9) Matrix Spike Duplicate (matrix-match commodity spiked at 10 ppm)
(10) Blank (ACN)
(11) Samples
(12) Blank (ACN)
(13) For every 5 samples analyzed, run a CCV vial to check for accuracy.
(14) CCV (end)
(15) Blank (ACN)

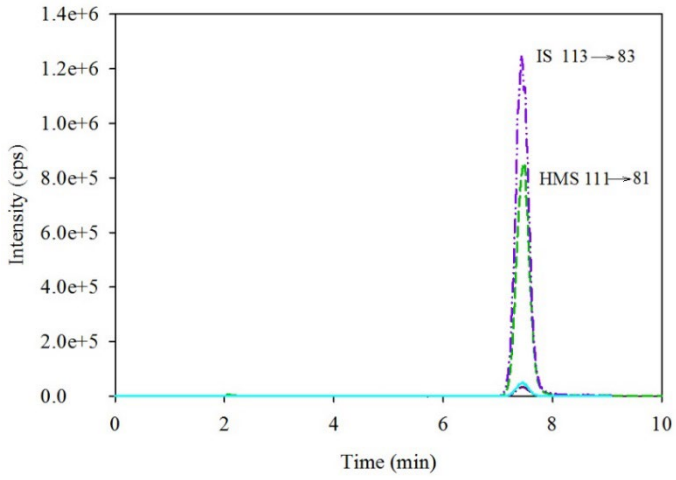
2023.8.3.3 Recommendation for Cleaning the LC column.

Cleaning and regeneration information can be found in the manufacturer's insert that is included with the column. This can be used if the backpressure increases or if your peak shape is degraded.

Wash column with:
30 column volumes of deionized water
30 column volumes of 0.5 M NaCl
30 column volumes of deionized water
After column wash is complete, re-equilibrate the column with the starting mobile phase. Be sure that the wash is diverted away from the mass spectrometer.

2023.9 CALCULATIONS

An example chromatogram is included below.



Instrument Software is used to prepare a standard curve from each day's data.

Concentrations obtained using the calibration curve are adjusted by the appropriate dilution factor (50 for solid samples and 10 for liquid samples if samples are prepared with no further dilution) and converted from Na_2SO_3 to SO_2 using the following calculation:

$$ppm\ in\ sample = \left(\frac{x\ \mu g\ Na_2SO_3}{mL\ LC\ vial}\right) * \left(\frac{1\ mL\ vial}{0.2\ mL\ extract}\right) * \left(\frac{df}{m}\right) * \left(\frac{64\ gmol^{-1}\ SO_2}{126\ gmol^{-1} Na_2SO_3}\right)$$

where *x* is the concentration (mg/kg) in vial from the calibration curve, *df* is the dilution fraction for the sample, *m* is the mass (g) of sample analyzed. The final term of the equation is used to convert from concentration Na_2SO_3 to SO_2. All values should be reported as µg SO_2/g food sample.

2023.10 QUALITY CONTROL AND CONFIRMATION CRITERIA

2023.10.1 Quality Control Criteria

(1) The r^2 values for all calibration curves must be ≥ 0.990.
(2) Matrix Spike and Matrix Spike Duplicate Recoveries: 80-120%, Relative Percent Difference (RPD%) must be < 20.
(3) ICV and CCV Recoveries: 90-110%
(4) Method Blank and Matrix Blank must be below the LOQ, as defined by the lowest calibration standard (0.05 µg/mL). If no acceptable matrix blank is available, a representative matrix may be used instead.

2023.10.2 Confirmation Criteria

(1) The relative abundance ratio of the confirmatory ion to the quantitation ion should be determined for each sample as well as the standards. The relative abundance ratio should match the comparison standard within ± 10% as outlined in the CVM Guidance for Industry 118.[6]
(2) The relative retention time matching should be ≤ 5% relative to the standard as outlined in the CVM Guidance for Industry 118.[6]
(3) The S/N ratio of the confirmation transition (111→80) on the lowest calibration point (0.05 µg/mL) must be ≥ 3

2023.10.3 Duplicate Analysis Requirements

For sulfite samples analyzed with the LC-MS/MS method, no check analysis is required. The samples should be analyzed in duplicate. A percent relative difference should be calculated for the two extracts and the percent difference should be less than 20%.

2023.11 Instrument Performance Criteria Evaluation

Instruments must achieve the following performance criteria to successfully run the method. Three replicates of the protocol below should be completed prior to implementing the method in your laboratory.

(1) Prepare a spike of the lowest calibration point (0.01 µg/mL Na_2SO_3 or 0.05 µg/mL Na_2SO_3).
(2) Analyze the samples using the parameters included in this compendial method on your instrumentation.

(3) Determine the signal to noise ratio of the confirmatory transition (111 → 80). This must be greater than or equal to 3.

2023.12 VALIDATION INFORMATION/STATUS

2023.12.1 Single Laboratory Validation

This was a level 2 validation conducted under the FDA Foods Program Guidelines[5]. A total of 12 different types of foods and beverages were evaluated. These include dried fruits and vegetables, frozen seafood, sweeteners, and juices. The method was validated at 5 concentrations (0.5, 5, 10, 15, 100 ppm SO_2) in 12 food matrices. Accuracy data showed spiked recoveries ranging from 84-115% with % RSDs ranging from 1-17%. Six commercially-available sulfited products were analyzed using the LC-MS/MS method, as well as the MW method, to determine if differences exist.

2023.12.2 Multi-laboratory Validation

This was a level 4 validation conducted under the FDA Foods Program Guidelines[5]. A multi-laboratory validation was conducted with 11 laboratories in the United States and Canada. Four matrices were spiked at varying concentrations and three additional commercially sulfited matrices were included. An abbreviated comparison study between the LC-MS/MS and Optimized Monier-Williams (OMW) was conducted for select samples. Average recoveries for all matrices ranged from 86-114% with % RSD_r and % RSD_R of 4.5-17.5 % and 8.6-22.5 %, respectively.

Data from the two validation studies are available in the cited publications. Raw data may be examined by contacting the study director.

2023.12.3 Single Laboratory Validation: Allium Containing Products

This was a level 2 validation conducted under the FDA Foods Program Guidelines.[5] A total of 4 matrices that could potentially contain *Allium* ingredients were spiked at 3 concentrations centered around the 10 ppm SO_2 regulatory threshold. Accuracy data showed spiked recoveries ranging from 102-116% with %RSD_r of 1-19%.

Allium containing product results: Average percent recovery (%RSD) for three concentration spikes from four representative food matrices of types of samples that could contain *Allium*.[1]

Matrix	5 ppm SO_2	10 ppm SO_2	20 ppm SO_2
Hummus	111 (1)	107 (3)	106 (3)
Phyllo Shells	105 (1)	102 (3)	107 (1)
Potato Chips	107 (13)	112 (13)	106 (10)
Quinoa	115 (19)	108 (16)	116 (11)

[1]n=3, % RSD is shown in parenthesis

2023.12.4 Single Laboratory Validation : High Moisture Solids

The validation study consisted of analyzing canned peaches, canned apricots, canned tuna in water, canned tuna in oil and jarred pickles. All samples were extracted at 0x, 1/2x, 1x, and 2x the regulatory labeling threshold level of 10 ppm SO_2. The recoveries were blank corrected during the calculation due to the presence of natural sulfites in these products. All samples were analyzed in triplicate spread out across three days of analysis. Jarred pickles were found to be analyzed better using the liquid modification. This matrix was validated since it had not been included in other validations to date.

2023.12.4.1 High Moisture Modification Results

Average percent recovery and $\%RSD_r$ for three concentration spikes from four representative high moisture solid food commodities.

Matrix	5 ppm SO_2	10 ppm SO_2	20 ppm SO_2
Canned Peaches	108 (8)	104 (5)	100 (4)
Canned Apricots	99 (7)	104 (3)	97 (1)
Canned tuna in oil	92 (10)	91 (4)	88 (3)
Canned tuna in water	86 (4)	89 (3)	89 (2)

2023.12.4.2 Liquid Modification for Peppers Results

Average percent recovery and $\%RSD_r$ for three concentration spikes for a representative pepper food commodity.

Matrix	5 ppm SO_2	10 ppm SO_2	20 ppm SO_2
Jarred Peppers	113 (5)	108 (6)	104 (11)

2023.12.5 Matrix extension data

Average percent recovery for different sample commodities spiked at 10 ppm SO_2 in duplicate.

Matrix	Protocol	Recovery 1	Recovery 2
Dried Banana Chips	Low Moisture	81	81
Sundried Tomatoes	Low Moisture	81	85
Sugar Palm Fruit	Basic protocol	100	104
Chutney 1	Basic protocol	121	119
Chutney 2	Basic protocol	110	117
Masala Powder	Low Moisture	106	108
Gummy Bears	Basic protocol	98	92
Pecans	Coconut	104	101
Sesame Brittle	Coconut	111	115
Peanut Brittle	Coconut	114	116
Smoked Salmon	Coconut	100	100
Preserved Radish	Basic protocol	107	103
Halawa	Coconut	109	115
Red Melon Seeds	Basic protocol	111	111
Mini muffins	Basic protocol	117	119
Energy Drink	Liquid	108	100
Chocolate Cereal	Basic protocol	95	93
Saltine Crackers	Low moisture	100	105
Coconut Cookies	Low moisture	106	96
Diced White Potatoes (canned in water)	Basic	113	113
Soybean Paste	Basic	113	112
Flour Tortillas	Basic	106	112

2023.12.6 Method Performance Metrics

2023.12.6.1 Accuracy and Precision Data

Use of this method for the determination of sulfites in food and beverage samples has been previously validated with acceptable results[1-2]. Per the guidelines of the FDA Foods Program for the initial validations,[5] acceptable recovery percentages should fall between 80 and 120% with $\%RSD_r$ less than 16%.

2023.12.6.2 Method Detection Limits

Limit of detection (LOD) and limit of quantitation (LOQ) determinations were difficult due to the presence of a blank response in both the matrix and method blanks. The background concentration in the method blanks is due to the presence of sulfite in both the formaldehyde and labeled stable isotope (purity = 95%). Because this background concentration should be consistent for all samples, the standard curve corrects for the increased area. However, all of the samples investigated in the single lab validation had an additional concentration of background sulfite.

Because the signal-to-noise ratio is >10 for any injected sample, the MDL was calculated by analyzing seven blank samples for each matrix studied. The mean (μ_b) and standard deviation (σ_b) of these analyses were calculated, and the MDL was determined using the

following formula: MDL = $\mu_b + 3.3\sigma_b$. The MDL was determined for six different matrices to determine the expected range for common sulfite matrices.

Food Matrix	Blank Concentration[a] (ppm of SO_2)	MDL[b] (ppm of SO_2)
White grape juice	0.08	0.12
Molasses	0.36	0.67
Dried potatoes	0.17	0.27
Crystallized ginger	0.12	0.30
Dried apricots	0.40	0.75
Frozen raw shrimp	0.59	0.72

[a]Calculated concentration of seven method blanks in food matrix.
[b]MDL was calculated using the following formula: MDL= $\mu_b + 3.3\sigma_b$, where μ_b is the mean blank concentration for seven method blank replicates and σ_b is the standard deviation for these blanks.

The MDL varied from 0.12 ppm in white grape juice to 0.75 ppm in dried apricots, all of which are >10 orders of magnitude below the 10 ppm SO_2 regulatory threshold. This shows that this method is fit for purpose for analyzing regulatory samples and there is no need for this calculation to be repeated in each implementing laboratory. However, laboratories should pay close attention to the Instrument Performance Criteria Evaluation section (2023.11) for guidance on ensuring instrument suitability for performing the method.

2023.12.6.3 Measurement Uncertainty

Measurement uncertainty was calculated using the formula described in ORA LAB 5.4.6. Briefly, the % RSD was calculated for all 180 single lab validation samples (spikes) and the following formula was used:

$$Measurement\ Uncertainty\ (U) = k(RSD)$$

Where k = $t_{(df=n-1,\ 95\%\ confidence\ interval,\ two\text{-}sided)}$

For this analysis, n=180, k= 1.97 for n-1=179, and % RSD = 10.31.
U = (1.97)*10.31 = 20.3 %

2022.13 REFERENCES

(1) Robbins, K. S.; Shah, R.; MacMahon, S.; and de Jager, L. S. 2015. Development of a Liquid Chromatography-Tandem Mass Spectrometry Method for the Determination of Sulfite in Food. *J Ag Food Chem* .

(2) Carlos, K. S.; de Jager, L. S. 2017. Determination of Sulfite in Food by Liquid Chromatograph Tandem Mass Spectrometry: Collaborative Study. *J. AOAC Int.* https://doi.org/10.5740/jaoacint.17-0033

(3) Carlos, K. S.; Conrad, S. M.; Handy, S. M.; de Jager, L. S. Investigation of food products containing garlic or onion for a false positive sulphite response by LC-MS/MS. *Food Addit. Contam. Part A*.

(4) Robbins Carlos, K. S.; de Jager, L. S. 2016. Comparison of multiple methods for the determination of sulphite in Allium and Brassica vegetables. *Food Addit. Contam. Part A.* 33 (10), 1509-1517.

(5) FDA Guidelines for the Validation of Chemical Methods for the FDA Foods Program;3rd edition, https://www.fda.gov/food/laboratory-methods-food/foods-program-methods-validation-processes-and-guidelines

(6) FDA Center for Veterinary Medicine. Guidance for Industry 118, 2003. Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues; https://www.fda.gov/downloads/AnimalVeterinary/GuidanceComplianceEnforcement/GuidanceforIndustry/UCM052658.pdf