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.

The test sample is mixed with a 0.2% formaldehyde extracting 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 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. The HMS ions are identified by retention time and ion ratio matching with the calibration standards. The identification criteria required is detailed in CVM 118- Guidance for Industry – Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues54. Quantitation was performed using calibration standards in solvent. The concentration of sodium sulfite was 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 these ratios for calibration standards 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 C-004.03 replaces version C-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 C-004.02 replaces version C-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 2021 (2021)

Author: Katie Carlos

CFSAN/ORS reviewers: Lowri de Jager, Tim Begley

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2021.1 METHOD TITLE: Determination of Sulfites in Food using Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS)

2021.2 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:

Matrices	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
Brassica genera: cabbage, broccoli, cauliflower, kale, Brussels sprouts	Single lab investigation[3]	2016	POC: Katie Carlos
Allium genera included on ingredient list (not pure *Allium*): hummus, quinoa, baked phyllo shells, and potato chips	Single lab matrix extension[4]	2020	POC: Katie Carlos
High moisture matrices: canned peaches, canned apricots, canned tuna (in both oil and water), jarred pickles.	Single lab validation	2019	POC: Katie Carlos
Pure *Allium* genera	Single lab investigation[3,4]	2016 and 2020	POC: Katie Carlos
Dried Banana Chips low moisture modification	Single lab verification	2021	POC: Andrea Heise
Sundried tomatoes low moisture modification	Single lab verification	2021	POC: Katy Niehaus
Sugar Palm Fruit (Kaong) Basic protocol	Single lab verification	2021	POC: Katy Niehaus
Chutney Basic protocol	Single lab verification	2021	POC: Nariman Patenaude

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.

This 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 8 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 multilaboratory validation did not include the *Brassica* genera (cabbage, broccoli, cauliflower, kale, etc.) . However, a thorough investigation of the blank concentration in 5 different Brassica vegetables (cabbage, broccoli, cauliflower, kale, Brussels sprouts) was conducted[3]. All of the vegetables had SO_2 concentrations below 5 ppm. Due to this data we feel that the method can be extended to include the Brassica genera. 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 SLV: five different matrices were spiked at three concentrations.

2021.3 PRINCIPLE

The test sample is mixed with a 0.2% formaldehyde extracting 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 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. The identification criteria required is detailed in CVM 118- Guidance for Industry – Mass Spectrometry for Confirmation of the Identity of Animal Drug Residues[5]. Quantitation was performed using calibration standards in solvent. The concentration of sodium sulfite was 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 these ratios for calibration standards 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.

2021.4 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 MLV, laboratories used equivalent reagents and achieved equivalent 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)

2021.5 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).

2021.6 PREPARATION OF SAMPLES OR TEST PORTIONS

2020.6.1 Prepare 2.0% Formaldehyde Extracting Solution

(1) Accurately weigh 1.925 g ammonium acetate onto a piece of weighing paper.
(2) Quantitatively transfer the ammonium acetate to the 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. 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.

2021.6.2 Prepare 0.2% Formaldehyde Extracting Solution

(1) Using the graduated cylinder, transfer 100 mL of the 2.0% formaldehyde extracting solution to the 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 2 weeks in a sealed glass container at room temperature.

2021.6.3 Prepare 10 mg/mL Na_2SO_3 Standard Stock Solution

(1) Using the weighing paper and 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 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 20 mL amber glass vial and store at 4°C. This solution can be stored for up to 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 of the formaldehyde.

2021.6.4 Prepare 25 ppm $Na_2{}^{34}SO_3$ Internal Standard Stock Solution

(1) Using the weighing paper and 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 expiration dates longer than those stated here if they desire but the response of the internal standard must compare within 50% to the initial response observed with the fresh standard.

2021.6.5 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).

2021.6.6 Prepare 1 M ammonium acetate solution

(1) Using the weighing paper and 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.

2021.6.7 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.

2021.6.8 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 thoroughly mix.

2021.6.9 Sample Preparation

General: Samples should be stored at -20°C until analysis. On the day of analysis, samples should be removed from the freezer and allowed to thaw. They 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.

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 Waring 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.

2021.6.10 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 2021.6.13.

2021.6.11 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 Waring 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.

2021.6.12 Modifications for Low-Moisture Dried Vegetables

(1) Optional step: If the sample is not a fine powder, grind the entire sample using the Waring blender.
(2) Weigh out 5.00 ± 0.25 g of dried vegetable directly into a 50 mL centrifuge tube.
(3) Add 30 mL of 0.2 % formaldehyde solution to centrifuge tube and place on a tube rotator at 70 rpm for 10 min.
(4) Transfer tubes to a beaker and sonicate for 8 minutes.
(5) Remove tubes from the sonicator and centrifuge at 4000 rcf for 5 min.
(6) Decant supernate into 50 mL stoppered graduated cylinder.
(7) Add an additional 20 mL of 0.2% formaldehyde solution to original falcon tube and repeat extraction steps (3-5).
(8) Add supernate to stoppered graduated cylinder and dilute to 50 mL with 0.2% formaldehyde solution.

(9) Continue with the SPE clean-up and heating derivatization as described in step 2021.6.13.
(10) Note: This is listed in the filtering section below but use 0.45 µm filter (yellow) instead of 0.20 µm filter (blue).

2021.6.13 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, we 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.
(4) Once the dropping has stopped, turn off the vacuum and remove the beaker. Transfer the extract to the 50 mL stoppered graduated cylinder.
(5) Add an additional 20 mL of 0.2% formaldehyde solution to original falcon tube and repeat extraction steps (rotator, sonicator, and centrifuge).
(6) Repeat filtering through the empty cartridge. Add additional extract to the same stoppered centrifuge tube rinsing with additional 0.2% formaldehyde solution.
(7) Continue with the SPE clean-up and heating derivatization as described in step 2017.6.13 but reduce the volume of extract used to 1.5 mL.

2021.6.14 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°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.

2021.7 APPARATUS/INSTRUMENTATION

(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 (BSM version 1.40.1248) and a sample manager (SM version 1.40.2532).
(9) Mass spectrometer. – Applied Biosystems 4000 Q-trap LC-MS/MS system with electrospray source in the negative ion mode using Analyst version 1.5.2 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). Column is thermostatted at 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)

2021.8 METHOD

2020.8.1 Prepare standard curve for LC-MS/MS analysis (THIS SHOULD BE DONE DAILY)

(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

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)
0.8	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

2021.8.2 Prepare samples for LC-MS/MS analysis

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

2021.8.3 LC-MS/MS Analysis

General: The MS/MS data was 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 analysis. Agilent 1290 Infinity and Shimadzu has also been used for some samples. Slight adjustments to dilution factor and declustering potential may be necessary if a different MS system is utilized.

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 be tuned slightly differently. If this is the case and the responses are significantly higher or lower than what can be expected, retune the CE for the quantitation ion to make adjustments in the sensitivity. Authors can be contacted for assistance with this issue.

MS/MS Conditions for the Monitored Transitions on a 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

MS/MS Conditions for the Monitored Transitions on a 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

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%

Time Profile for the External Switching Valve

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

i. The following conditions are for the 4000 Q-trap:
    1. Curtain gas: 35 au
    2. Collisionally activated dissociation (CAD) gas: medium
    3. Ion spray voltage: -1200 V
    4. Source temperature: 550 °C
    5. Gas 1 pressure: 70 au
    6. Gas 2 pressure: 40 au
    7. Injection volume: 5 µL
    8. Column temperature: 30 °C
    9. Flow rate: 0.30 mL/min

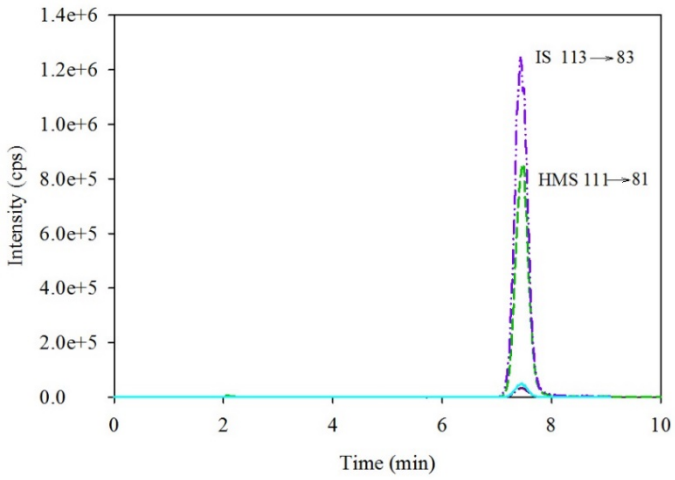
ii. The following conditions are for the 5500 Q-trap:
    1. Curtain gas: 35 au
    2. Collisionally activated dissociation (CAD) gas: medium
    3. Ion spray voltage: -1200 V

4. Source temperature: 550 °C
5. Gas 1 pressure: 70 au
6. Gas 2 pressure: 40 au
7. Injection volume: 5 µL
8. Column temperature: 30 °C
9. Flow rate: 0.30 mL/min

(2) Run the samples using the following template:

i. Blank (ACN) injection
ii. Standard curve
iii. Blank (ACN) injection
iv. Samples
v. For every 5 samples analyzed, run a mid-point calibration vial to check for accuracy

2021.9 CALCULATIONS



An example chromatogram is included below.

Analyst software is used to prepare a standard curve from each day's data. Adjustments are then made for the dilution, weight of the sample, and conversion from sodium sulfite to sulfur dioxide using the following formula:

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

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.

2021.10 VALIDATION INFORMATION/STATUS

Use of this method for the determination of sulfites in food and beverage samples has been previously validated with acceptable results[1-2].

Single lab validation. This was a level 2 validation conducted under the FDA Foods Program Guidelines[6]. 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.

Multi-lab validation. This was a level 4 validation conducted under the FDA Foods Program Guidelines[6]. 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 OMW was conducted for select samples. Average recoveries for all matrices ranged from 86-114% with % RSDr and % RSDR of 4.5-17.5 % and 8.6-22.5 %, respectively.

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

Single lab validation: Allium containing products. This was a level 2 validation conducted under the FDA Foods Program Guidelines[6]. 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 %RSDr of 1-19%.

Average percent recovery 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

Single lab 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 was validated since it hasn't been included in other validations to date.

High Moisture Modification results. Average recovery and $\%RSD_r$ included here.

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)

Liquid modification for Peppers results. Average recovery and $\%RSD_r$ included here.

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

Single lab verification data. All spikes were completed at 10 ppm SO_2

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

2021.11 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* .

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(3) 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.

(4) 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*.

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(6) 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