FOOD COMPOSITION AND ADDITIVES

Validation of the Biofish-300 SUL Enzymatic Biosensor for the Detection of Sulfite in Crustacean

AOAC *Performance Tested Method*SM 031802

Abstract

Background: Sulfites are some of the oldest and most widespread preservatives in our food supply. They are food additives that have antioxidant properties, but they are also recorded as allergens by the main international regulatory bodies on food safety because of their adverse health effect. Hence, sulfites maximum concentration in foodstuff is regulated and they must be ensured by the agro-food processing industries. The most widely used technique for the quantification of sulfites is the Modified Monier-Williams (AOAC Official Method **990.28**). *Objective:* In this method, SO_2 is released from sulfites and some bound compounds when the sample is mixed with an acid (normally hydrochloric acid, but sometimes phosphoric acid) and heated. The SO_2 is distilled using a stream of nitrogen gas, which carries the gaseous SO_2 into an absorbing solution of hydrogen peroxide (H_2O_2) where it is oxidised to sulphuric acid. The amount of SO_2 distilled into the H_2O_2 is determined by titration with 0.1M sodium hydroxide. Apart from being time consuming (at least 2 h) and the usage of toxic solvents, the method presents some other disadvantages that make it inappropriate as a routine-control technique for the agro-food industry. Hence, the industry demands simple, fast and accurate methods for sulfite level monitoring. *Methods:* BIOLAN is a SME that develops and commercializes biosensors for quantitative analysis of food quality and safety parameters, based on its proprietary enzyme-based electrochemical biosensor technology platform. This technology enables high accurate and robust analysis with a compact device that help the users to control the quality in an easy and safety manner. Biofish-300 SUL method is a highly specific enzimatic biosensor for the rapid quantification of sulfite, measured as SO_2 content, in crustaceans. It consists on the extraction of sulfite in an aqueous based solution, by the aid of an Ultra-turrax or similar, and its subsequent quantification by the biosensor after previous calibration (3 min). *Results:* Sulfite in raw shrimp head-on, raw shrimp head-off, and boiled shrimp was analyzed, and performance was examined using naturally contaminated and spiked samples by comparisons with AOAC *Official Methods of Analysis*SM (OMA) **990.28**. Linearity, selectivity, matrix, consistency, and robustness were evaluated. All results were within acceptable ranges except robustness, which reflected deviation in the sample volume and ultraturrax time compared with the standard assay procedures described in the Biofish 300 SUL Instruction Manual. Accuracy, assessed as a comparison of the Biofish results with the OMA results, ranged from 82 to 115% in all samples except for fortified raw shrimp head-on, in which the low level yielded an accuracy of 138%. The method bias was in general negative in both incurred and fortified high levels, and slightly positive in incurred low levels. Repeatability was very good as shown by the low RSD_r values, demonstrating acceptable repeatability precision with results <10% in most of the evaluated values. Regression analyses showed a good correlation between the Biofish and OMA methods with $R^2 > 0.99$ in all cases. *Conclusions:* As a whole, accuracy, recovery and bias within range results indicate that the kit provides accurate and precise sulfite quantification for all the evaluated matrices, confirming that sample preparation and assay procedures produce acceptable results. Biofish 300 SUL has proved to be a suitable tool for monitoring sulfite levels in quality control routines due to its high accuracy, precision, rapid response and ease of use. *Highlights:* With a simple sample preparation, results are obtained in approximately 3 min, making a big difference with other technologies that require specific skills or tedious sample pretreatments and analysis procedures.

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The method was independently tested, evaluated, and certified by the AOAC Research Institute as a *Performance Tested Method*SM. *See* http://www.aoac.org/testkits/steps.html for information on certification.
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Scope of Method

(**a**) *Target analyte*.—Sulfite, measured as SO_2 content.

(**b**) *Matrixes*.—Raw shrimp head-on, raw shrimp head-off, and boiled shrimp.

(**c**) *Claims*.—Quantification of sulfite from 30 to 150 mg/kg and 50 to 300 mg/kg. Within these ranges, the Biofish method was equivalent to *Official Methods of Analysis*SM (OMA) **990.28**, the optimized Monier-Williams method for determination of sulfites in foods (1, 2). The method is not intended for regulatory use.

Intended Users

Food laboratories.—Technical staff of fish and aquaculture companies, including primary and processing sector.

Definitions

(**a**) *Linearity*.—The ability of the method to obtain test results proportional to the concentration. Linearity of the method extending beyond the set of standards or calibrators supplied with the kit.

(**b**) *Selectivity*.—Ability of the method to detect analytes without interference from matrixes or other components of similar behavior.

(**c**) *Recovery*.—The ratio of the mean method result to the true value, expressed as a percentage: [$\text{mean}_{\text{cand}}$ / known spike] × 100 or [mean_{ref} / known spike] × 100.

(**d**) *Accuracy*.—The ratio of the mean candidate method result to the reference method result, expressed as a percentage: [$\text{mean}_{\text{cand}}$ / mean_{ref}] × 100.

(**e**) *Bias*.—The difference between the candidate method mean result and the true value: $\text{mean}_{\text{cand}}$ – known spike.

(**f**) *Relative bias*.—The difference between the candidate method mean result and the mean reference method result: $\text{mean}_{\text{cand}} - \text{mean}_{\text{ref}}$.

(**g**) *Standard deviation of repeatability*.

$$s_r = \sqrt{\frac{\sum_{i-1}^{n}\left(X_i - \bar{X}\right)^2}{n-1}}$$

where X_i = the *i*th value; *Xbar* = the mean value; and n = the number of values.

(**h**) RSD_R.—$\text{RSD}_r = [s_r / \text{mean}_{\text{cand}}] \times 100$.

(**i**) *LOD*.

$$\text{LOD} = \frac{\bar{X}_0 + 3.3(s_b)}{1 - 1.65m}$$

where $\bar{X}_0$ = the mean analytical value of the known negative matrix; S_b = the intercept of the plot; and m = the slope of the plot.

(**j**) *LOQ*.—LOQ = 3 × LOD.

Principle of the Method

The Biofish-300 SUL is an enzymatic biosensor for the specific detection of sulfite. The test method consists of the extraction of sulfite in measurement solution and subsequent quantification by the biosensor after previous calibration. The enzymatic biosensor is a system that incorporates a biochemical sensing element (based on a specific oxidative enzyme) in intimate contact with or in close proximity of a transducer system that relates the concentration of an analyte to a measurable signal. The addition of the analyte causes sequential redox reactions that involve a release of electrons proportional to the concentration of the analyte (Figure 1). Thus, these biosensors combine the specificity and the selectiveness of enzyme-analyte reactions with highly sensitive electrochemical transduction.

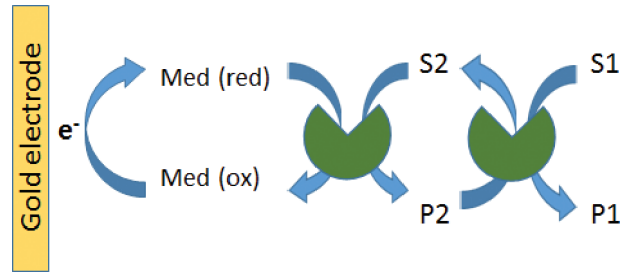

Figure 1. Redox reactions at the surface of the electrode.

General Information

Sulfites are some of the oldest and most widespread preservatives in our food supply. They were used in Greek and Roman times in wine, but it was only in the 1880s that their use as preservatives in meats was pioneered by Australian and South American beef producers wanting to ship their products to England. The use of sulfites in fruit and vegetables became common with the growth of the processed food industry in the twentieth century.

Sulfites are compounds that contain the sulfite ion SO_3^{2-}. Sulfites and sulfiting agents are widely used as additives in the food and pharmaceutical industries thanks to their preservative, antioxidant, and antimicrobial activity.

EU No. 1129/2011 (3) established a list of food additives and their limits in various matrixes including sulfites in seafood (Tables 1 and 2; 3).

Principle

Test Kit Information

(**a**) *Kit name*.—Biofish-300 SUL.

(**b**) *Cat. No*.—BF004.

(**c**) *Ordering information*.—*Worldwide*.—Biolan Microbiosensores S.L. Parque Tecnológico de Vizcaya, Laida Bidea, 409, Zamudio, Bizkaia, Spain 48170, Tel: +34-946-57-41-61, Website: www.biolanmb.com.

(**d**) *Test kit components*.—(*1*) *BTFSUL20*.—SUL Biotest for 20 analyses + SUL1 extraction reagent (SEFSUL1) for 20 analyses.

(*2*) *BTFSUL50*.—SUL Biotest for 50 analyses + SEFSUL1 for 50 analyses.

(*3*) *BTFSUL100*.—SUL Biotest for 100 analyses + SUL2 extraction reagent (SEFSUL2) for 100 analyses.

(*4*) *BTFSUL125*.—SUL Biotest for 125 analyses + SEFSUL2 for 125 analyses.

(*5*) *KITCFSUL*.—SUL calibration kit [four tubes with calibration reagent (CFSUL)].

Table 1. Unprocessed mollusks and crustaceans

E No.	Name	Maximum level, mg/L or mg/kg as appropriate	Restrictions/exceptions
E 220–228[a,b]	SO_2 — sulfites	150	Only fresh, frozen, and deep-frozen crustaceans and cephalopods; crustaceans of the *Penaeidae*, *Solenoceridae*, and *Aristaeidae* family up to 80 units
E 220–228[a,b]	SO_2 — sulfites	200	Only crustaceans of the *Penaeidae*, *Solenoceridae*, and *Aristaeidae* family between 80 and 120 units
E 220–228[a,b]	SO_2 — sulfites	300	Only crustaceans of the *Penaeidae*, *Solenoceridae*, and *Aristaeidae* family over 120 units

[a] Maximum levels are expressed as SO_2 and relate to the total quantity, available from all sources; an SO_2 content of not more than 10 mg/kg or 10 mg/L is not considered to be present.

[b] Maximum limits in edible parts.

Table 2. Processed fish and fishery products including mollusks and crustaceans

E No.	Name	Maximum level, mg/L or mg/kg as appropriate	Restrictions/exceptions
E 220–228[a,b]	SO_2— sulfites	50	Only boiled crustaceans and cephalopods
E 220–228[a,b]	SO_2— sulfites	135	Only boiled crustaceans of the *Penaeidae*, *Solenoceridae*, and *Aristaeidae* family up to 80 units
E 220–228[a,b]	SO_2— sulfites	180	Only boiled crustaceans of the *Penaeidae*, *Solenoceridae*, and *Aristaeidae* family between 80 and 120 units
E 220–228[a,b]	SO_2— sulfites	200	Only dried salted fish of the "Gadidae" species
E 220–228[a,b]	SO_2— sulfites	270	Only boiled crustaceans of the *Penaeidae*, *Solenoceridae*, and *Aristaeidae* family over 120 units

[a] Maximum levels are expressed as SO_2 and relate to the total quantity, available from all sources; an SO_2 content of not more than 10 mg/kg or 10 mg/L is not considered to be present.

[b] Maximum limits in edible parts.

(*6*) *KITMFSUL1*.—SUL1 measurement kit [eight tubes of measurement reagent (MFSUL1) for a total of 160 analyses].

(*7*) *KITMFSUL2*.—SUL2 measurement kit [five tubes of measurement reagent (MFSUL2) for a total of 450 analyses].

(*8*) *Reference electrode (ELEREFN)*.—Reference electrode and storage solution.

(*9*) *KITVFSUL*.—SUL verifying kit (four tubes of verifying reagent).

(**d**) *Additional supplies and reagents.—(1) Distilled water.*

(*2*) *Plastic tube*.—Falcon, 50 mL.

(*3*) Plastic test tube.—25 mL.

Apparatus

(**a**) *Biofish biosensor*.—BF300-004, Biofish 300 sulfite.

(**b**) *Analytical balance*.—Capable of measurement with two decimal places (grams).

(**c**) *Mincer*.

(**d**) *Sample homogenizer*.—Ultraturrax IKA T-25, or equivalent.

(**e**) *Adjustable pipettes*.—Capable of delivering 20–200 and 500–5000 µL.

Reference Materials

GSCCA 1/2016.—Peeled shrimp containing 116 mg/kg SO_2.

Safety Precautions

There are no major risks. Sulfites may cause allergy-like reactions, including sneezing, runny nose, or wheezing; asthma symptoms in people with underlying asthma; or, rarely, hives or anaphylaxis. The use of personal protective equipment including gloves, laboratory coat, and protective eyewear is recommended.

General Preparation

(**a**) *Biotest storage and hydration*.—The Biotests are dispatched at room temperature and vacuum packed with silica gel crystals to protect them from atmospheric humidity. Once delivered, store unopened between 3 and 8°C until the expiration date. Hydrate the Biotest prior to using (this process should be carried out at least 24 h prior to use).—(*1*) Discard the silica gel crystals from inside the tube.

(*2*) Fill the tube with SUL measurement solution until the Biotest is submerged.

(*3*) Add 20 µL SUL calibration reagent. Once hydrated, the Biotest must be stored vertically at a temperature between 3 and 8°C for a maximum of 15 days.

(**b**) *SUL measurement solution*.—The SUL measurement kit supplies eight or five tubes to prepare the SUL measurement solution necessary to hydrate the Biotest and to carry out the measurements.—(*1*) Dissolve the contents of one tube of MFSUL1 in 250 mL distilled water, or dissolve the contents of one tube of MFSUL2 in 1 L of distilled water.

(*2*) Mix well. Store at room temperature and use within 15 days.

(**c**) *SUL extraction solution*.—The SUL extraction solution is necessary to prepare CFSUL and standards as well as to extract the sulfite from the matrix to be analyzed.—(*1*) Dissolve the

Table 3. Linearity in 30–150 ppm and 50–300 ppm ranges

30–150 ppm					50–300 ppm				
Sodium sulfite Std.	Biofish result	Mean	SD	Residual	Sodium sulfite Std.	Biofish result	Mean	SD	Residual
0	0	0	0	0.035	0	0	0	0	0.464
	0					0			
	0					0			
	0					0			
10	7	8	0.5	0.747	30	23	24	1.0	0.942
	8					25			
	8					23			
	8					23			
30	26	28	1.7	0.424	50	47	46	1.9	0.384
	28					45			
	27					47			
	30					43			
50	47	48	1.7	0.198	100	99	97	1.7	–0.562
	50					98			
	46					97			
	47					95			
100	95	98	3.1	–0.610	150	142	143	3.8	–0.643
	102					144			
	97					147			
	96					138			
150	148	149	2.9	–1.903	300	294	285	6.7	–1.680
	153					285			
	147					278			
	147					283			
300	285	285	10.3	1.108	600	525	535	17.1	1.095
	299					533			
	275					521			
	280					559			

contents of one tube of SEFSUL1 in 1 L water, or dissolve the contents of 1 tube of SEFSUL2 in 2 L water.

(*2*) Mix well. Store at room temperature and use within 15 days.

(d) *SUL calibration reagent*.—The SUL calibration kit supplies four tubes of powdered calibration reagent along with two empty tubes to prepare the sulfite standards. Add 10 mL SEFSUL to one tube of CFSUL and mix. To prevent loss of reagent, introduce the pipette tip through the perforated stopper when adding the extraction solution volume. Store at a refrigerated temperature (3–8°C) and use within 7 days.

(e) *Sulfite standard*.—The sulfite standards are prepared daily by diluting the calibration reagent, and this is the reagent used to calibrate the equipment.—(*1*) Prepare SUL standard 1 (range 30–150 mg/kg) by diluting 53.4 µL CFSUL to a final volume of 20 mL with SEFSUL extraction solution.

(*2*) Prepare SUL standard 2 (range 50–300 mg/kg) by diluting 100 µL CFSUL to a final volume of 20 mL with SUL extraction solution.

(f) *ELEREFN*.—Store in 4 M KCl at room temperature in the dark and in an upright position until use.

(g) *Biosensor setup*.—(*1*) Couple both the Biotest (inserting it into the Biotest carrier and tightening it with the screw) and the reference electrode into their respective connectors (Figure 2), ensuring that they are well connected. For safety reasons, it is recommended to never disconnect the reference electrode. When the equipment is disassembled after analyses are completed, the reference electrode remains protected with the Storage Solution supplied (4 M KCl).

(2) Fill the measurement cuvette with 10 mL SUL measurement solution and place it in the cuvette so that all components in the electrochemical cell are submerged (Figure 2). Connect the equipment to a 100–240 volts alternating current stable current power outlet and press the power button located next to the screen for 2 s. The menu main screen will appear (Figure 3a).

(*3*) In the BIOTEST option, Select the parameter to be analyzed, press », enter the Biotest Code (this code is located on the Biotest identifying label), press », and finally, select the measurement range with which you are going to work and press SAVE (Figure 3b).

(*4*) The analysis option on the main screen leads to the analysis menu: Recondition, Activate, Calibrate, Standard, Verifying Solution, Turrax, Bath, and Stand By (Figure 3c).

(h) *Activation and calibration of the Biotest*.—Once per day, before beginning to perform the analysis, the biotest must be

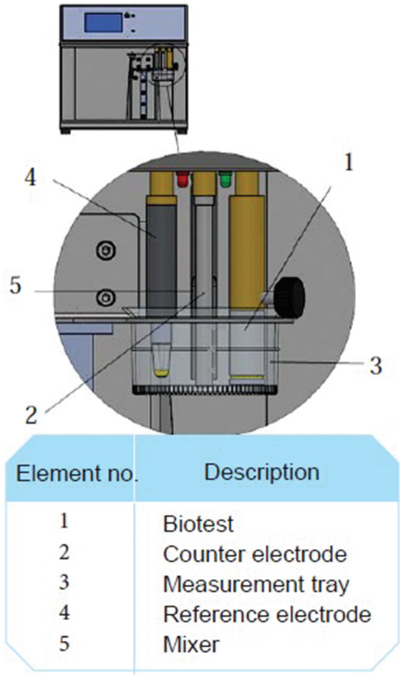

Figure 2. Assembly of the electrochemical cell.

reconditioned, activated, and calibrated.—(*1*) *Reconditioning*.—(*a*) Press "Recondition," verify the proper placement of the cuvette with the 10 mL SUL measurement solution, add sulfite standard (2 mL SUL standard 1 in case of 30–150 mg/kg measurement range or 1 mL SUL standard 2 in case of 50–300 mg/kg measurement range) into the measurement cuvette, and press CONTINUE.

(*b*) Wait for the equipment to emit an acoustic signal, press CONTINUE, and once again inject sulfite standard (2 mL of SUL standard 1 in case of 30–150 mg/kg measurement range or 1 mL SUL standard 2 in case of 50–300 mg/kg measurement range) into the measurement cuvette and wait for the process to end.

(*c*) The message "Biotest reconditioned. Ready to activate" will appear on the screen.

(*d*) Replace the cuvette's contents with 10 mL new measurement solution.

(*2*) *Activation*.—(*a*) Press "activate," verify the proper placement of the cuvette, and press CONTINUE to begin the activation.

(*b*) Wait for the equipment to emit an acoustic signal, press CONTINUE and inject sulfite standard (2 mL SUL standard 1 in case of 30–150 mg/kg measurement range or 1 mL SUL standard 2 in case of 50–300 mg/kg measurement range) into the measurement cuvette, and wait for the process to end.

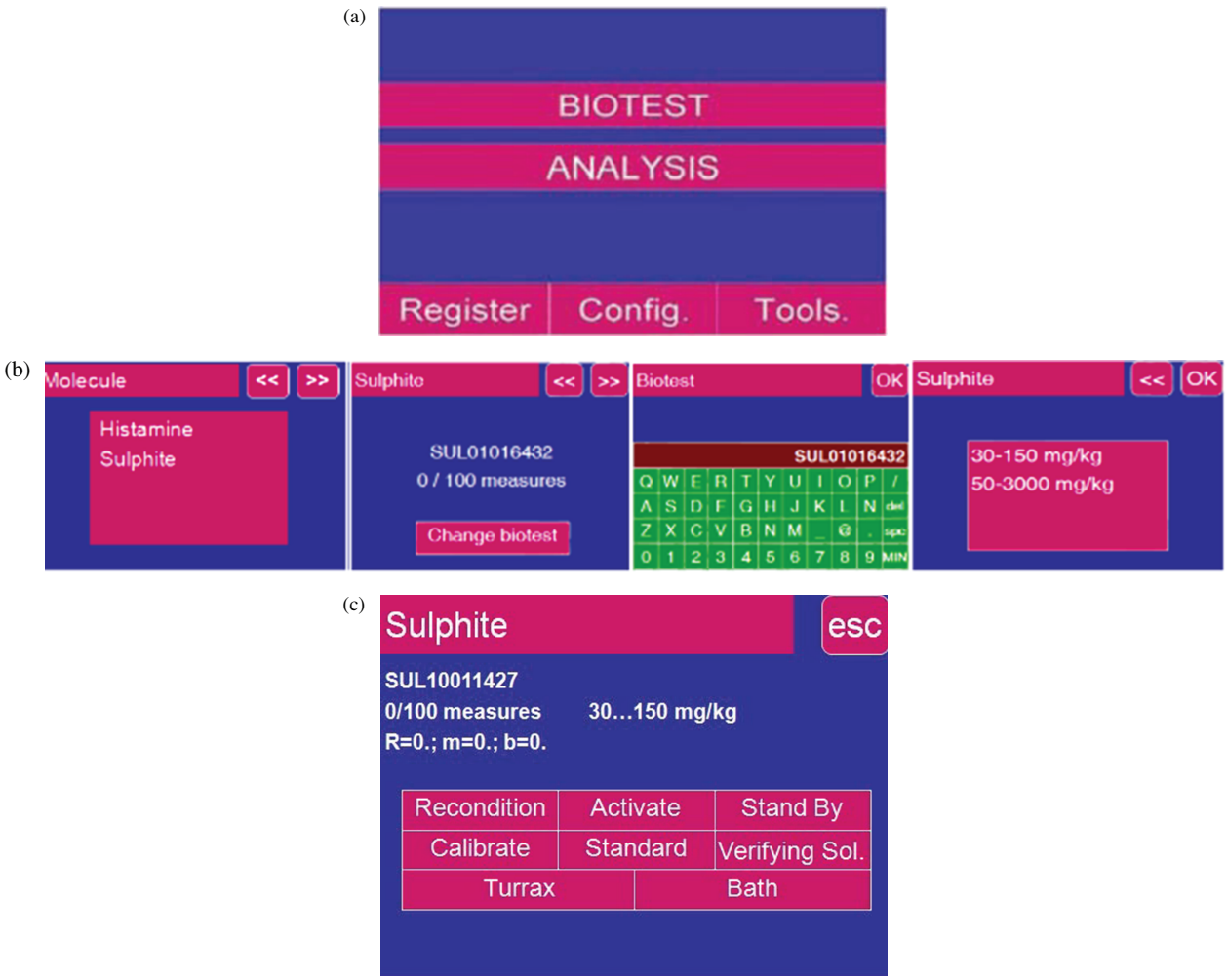

Figure 3. (a) Main menu screen, (b) screens of the Biotest option, and (c) screen of the analysis option.

(*c*) The message "Biotest activated. Ready for calibration" will appear on the screen.

(*d*) Replace the cuvette's contents with 10 mL new measurement solution.

(*3*) *Calibration*.—The calibration is carried out by making two additions of the sulfite standard to the measurement cuvette. To plot the calibration line, the equipment will take into account the standard, the variation in intensity brought about by the initial addition of the sulfite standard, and the sum of the variations in intensities brought about by both additions.—(*a*) Press calibrate, verify the proper placement of the cuvette with 10 mL of SUL measurement solution, and press CONTINUE.

(*b*) Wait for the equipment to emit an acoustic signal. Press CONTINUE and add sulfite standard into the measurement cuvette (2 mL SUL standard 1 in case of 30–150 mg/kg measurement range or 1 mL SUL standard 2 in case of 50–300 mg/kg measurement range). This is addition 1. *Note*: It is important to add the calibration standard immediately after pressing the CONTINUE button to record the current intensity correctly at the time of adding it.

(*c*) Wait for the equipment to register the change in intensity and emit an acoustic signal again. Press CONTINUE and add sulfite standard again (2 mL SUL standard 1 in case of 30–150 mg/kg measurement range or 1 mL SUL standard 2 in case of 50–300 mg/kg measurement range). This is addition 2.

(*d*) Wait until the parameters of the calibration appear on the screen together with a message of "calibration OK" or "calibrate again."

(*e*) Replace the cuvette's contents with 10 mL new SUL measurement solution (Figure 4).

Because of the calibration, the parameters of the linear regression obtained by the interpolation of the two calibration points and the 0.0 point will appear. *R*-values of at least 0.9985 and m-values ≤ –100 000 are deemed as valid to proceed with the analysis. If the regression is not valid ("calibrate again" appears), change the measurement cuvette, put it on standby for 30 s, and calibrate again. If the regression is valid ("calibration OK" appears), continue to *Standard Measurement*.

(*4*) *Standard measurement*.—The calibration status should be tested using the STANDARD option after every 15 measurements or after the device has not been running for 2 h.—(*a*) Press Standard in the analysis option, check that the cuvette is correctly fitted and filled with SUL measurement solution, and press CONTINUE to begin the standard measurement.

(*b*) Wait until the device records the measurement target and it issues a sound signal. Press CONTINUE and add the calibration standard (2 mL SUL standard 1 in case of 30–150 mg/kg measurement range or 1 mL SUL standard 2 in case of 50–300 mg/kg measurement range) into the measuring cuvette.

(*c*) Wait until the "Biosensor calibrated" or "Biosensor recalibrate" message appears.

(*d*) Replace the contents of the cuvette with 10 mL new SUL measurement solution. If "Biosensor calibrated" message appeared, proceed to *Sample Preparation*. If "Biosensor recalibrate" message appeared, return to step **3(a)**.

Sample Preparation

(**a**) *Preparation of crustacean (raw and boiled)*.—(*1*) Clean and dismantle the carapace and cephalothorax and other nonedible parts of the exoskeleton and the visible digestive tract (leave the hepatopancreas in case its measurement is required).

(*2*) Homogenize the sample with the aid of a mincer.

(*3*) Add 2 g homogenized sample to a plastic tube.

(*4*) Add 18 mL SUL extraction solution.

(**b**) *Sample extraction.—(1)* Homogenize the contents of the plastic tube with the aid of an ultraturrax (8000–9000 rpm for 15–20 s).

(*2*) Leave to stand for approximately 15 min at room temperature.

(*3*) *Proceed to analyze in Turrax mode*.—Sample extractions without hepatopancreas are stable up to 4 h at room temperature. On the other hand, sample extractions with hepatopancreas are stable only for 1 h at room temperature.

(**c**) *Analysis*.—(*1*) Press the option Turrax.

(*2*) Check that the cuvette is correctly fitted and filled with SUL measurement solution and press CONTINUE to begin the sample measurement.

(*3*) Wait until the device records the measurement target and it issues a sound signal. Press CONTINUE and pipette the clear supernatant of the extracted sample (2 mL in case of 30–150 mg/kg measurement range or 1 mL in case of 50–300 mg/kg measurement range) into the measuring cuvette.

(*4*) Wait until the process ends and the device shows the result of the analysis.

(*5*) Replace the contents of the cuvette with 10 mL new measurement solution.

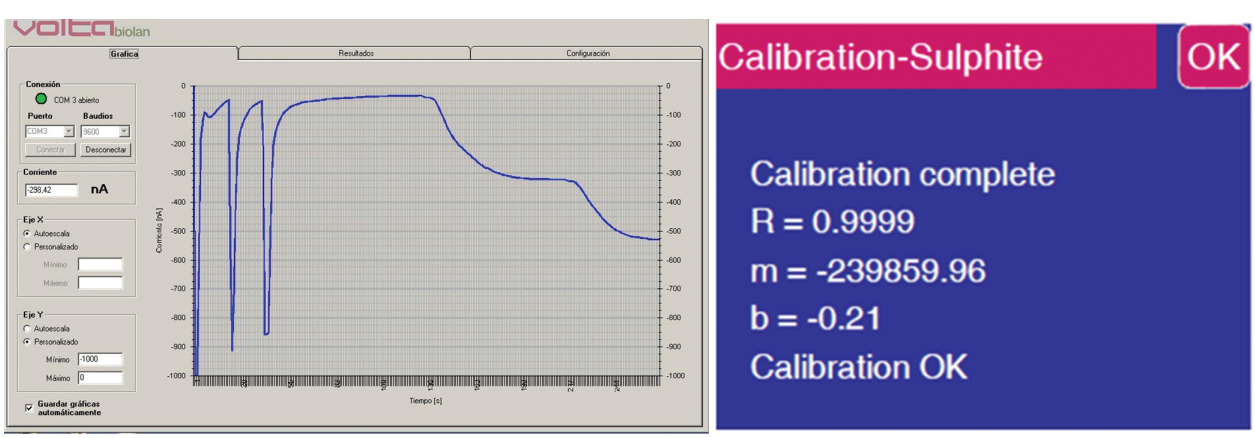

Figure 4. Evolution of the electric current during calibration and screen of calibration parameters obtained.

Interpretation and Test Result Report

The Biosensor expresses the analysis result in milligrams of sulfite (measured as SO_2) per kilogramms of crustacean, considering the dilution involved in the extraction of the sample.

(*1*) To save the result of an analysis, press SAVE.

(*2*) To view the saved results, enter the RECORDS option. Saved results can be downloaded to a computer.

(*3*) To delete the saved results, go to RECORDS and press DELETE ALL.

Validation Study

This validation study was conducted under the AOAC Research Institute (RI) *Performance Tested Method*SM (PTM) program. The Biofish-300 SULFITE method was validated for raw shrimp head-on, raw shrimp head-off, and boiled shrimp. The method developer studies included linearity, LOD, LOQ, bias, recovery and precision, selectivity, lot-to-lot consistency, stability, and robustness. Anfaco Cecopesca conducted an official analysis with OMA and statistical analysis. The independent laboratory study included bias, accuracy, repeatability, precision, LOD, and LOQ. Statistical analyses were performed by Microsoft Excel 2010 and SPSS statistics version 23.

Method Developer Studies

Linearity Study

(**a**) *Methodology*.—A calibration line with seven concentrations (including the blank) of sodium sulfite and four replicates per concentration level were analyzed. A stock solution of 3000 ppm sodium sulfite was prepared. Sodium sulfite standard (59 mg) was dissolved in 10 mL distilled water. Working solutions were prepared from this stock standard solution.—(*1*) *For 30–150 ppm*.—0, 10, 30, 50, 100, 150, and 300 ppm.

(*2*) *For 50–300 ppm*.—0, 30, 50, 100, 150, 300, and 600 ppm.

These solutions were prepared daily and stored at a refrigerated temperature (3–8°C). The standard solutions were measured in the Biofish system according to the *Analysis* step detailed above.

(**b**) *Results*.—The results are presented in Table 3. Linearity and residual plots for the different calibration ranges are presented in Figure 5–8. The results obtained show that the method is linear. The residual plots appear to have a sigmoidal pattern or concave if the zero point is disregarded. In either case, the patterns look nonrandom, but the residuals are all <10%, varying from 0.2 to 3.9% for the 30–600 ppm range and 0.4 to 9.3% for the 10–300 ppm range. Thus, the response is very close to linear.

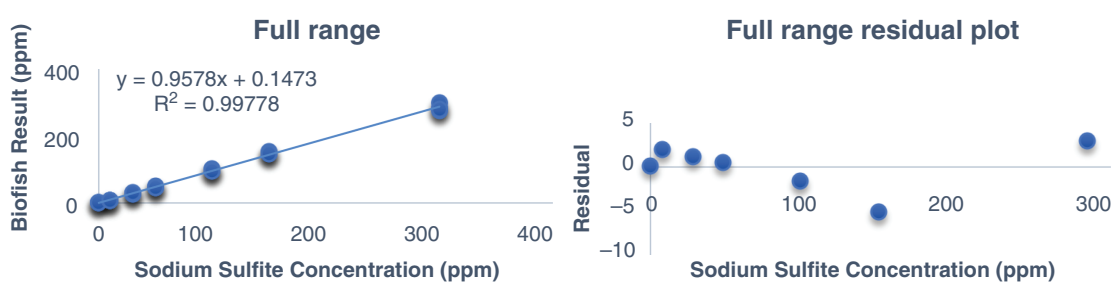

Figure 5. Linearity and residual plots for the full range of standards examined.

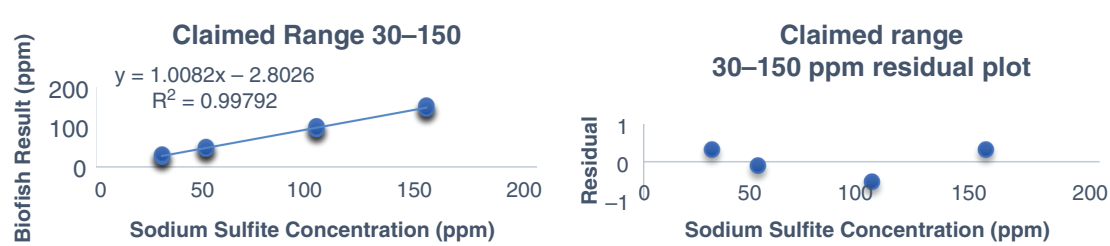

Figure 6. Linearity and residual plots for the claimed range of calibration (30–150 ppm).

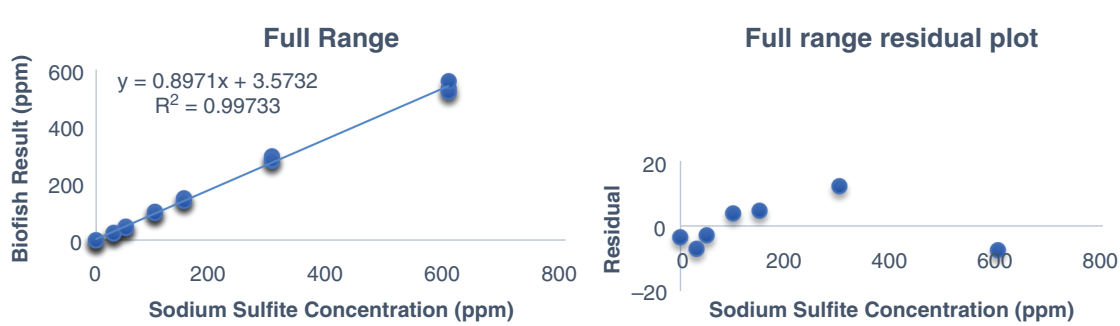

Figure 7. Linearity and residual plots for the full range of standards examined.

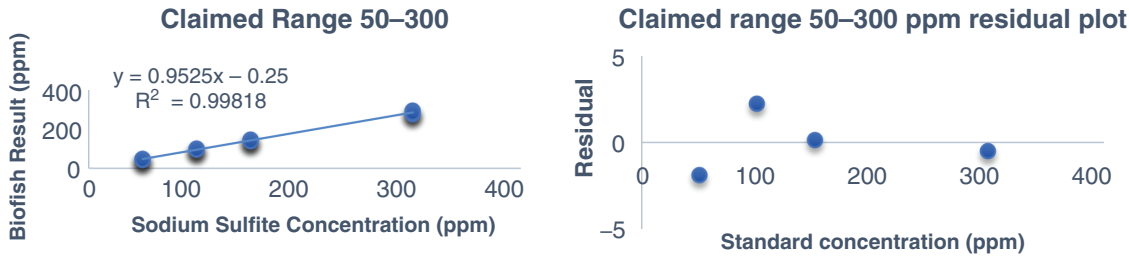

Figure 8. Linearity and residual plots for the claimed range of calibration (50–300 ppm).

Selectivity

(**a**) *Methodology*.—Potential interfering compounds were tested in sulfite-free extracts of each shrimp matrix at 100 ppm measured as SO_2 in SUL extraction solution. Compounds included the following: sodium sulfite, sodium bisulfite, sodium metabisulfite, potassium metabisulfite, formaldehyde-bisulfite adduct, methionine, and cysteine. Stock solutions were prepared.—(*1*) *Sodium sulfite (99%, S/6840/53; Fisher Chemical)*.—59 mg in 10 mL extraction solution (3 g/L SO_2).

(*2*) *Sodium bisulfite (64.7% SO2, 7631-90-5; Acros Organics)*.—16.2 mg in 10 mL extraction solution (1 g/L SO_2).

(*3*) *Sodium metabisulfite (97–100%, 7681-57-4; Scharlau)*.—14.8 mg in 10 mL extraction solution (1 g/L SO_2).

(*4*) *Potassium metabisulfite (96%, 16731-55-8; Sharlau)*.—17.4 mg in 10 mL extraction solution (1 g/L SO_2).

(*5*) *Formaldehyde-bisulfite adduct (95%, 112704; Sigma)*.—66.1 mg in 10 mL of extraction solution (3 g/L SO_2).

(*6*) *Methionine (L-Methionine 98%, M9625; Sigma-Aldrich)*.—23.3 mg in 10 mL extraction solution (1 g/L SO_2).

(*7*) *Cysteine (L-Cysteine 97%, 168149; Sigma-Aldrich)*.—18.9 mg in 10 mL extraction solution (1 g/L SO_2).

A 2 g portion of each matrix was weighed and spiked with the corresponding stock solution of the evaluated compound to obtain a final concentration of 100 ppm SO_2 in the matrix. The samples were extracted and measured with the Biofish system according to the *Analysis* step of sample preparation detailed above.

(**b**) *Results*.—The results obtained for the selectivity study are shown in Table 4. Because of solubility problems, calcium sulfite could not be evaluated. Calcium bisulfite and potassium bisulfite could not be acquired and therefore could not be evaluated.

No matrix effects or cross-reactivity to sulfur-containing amino acids were found.

Matrix Study

(**a**) *Methodology*.—The Biofish-300 SUL method was compared with OMA **990.28**, the optimized Monier-Williams method for determination of sulfites in foods (1, 2) for calculation of accuracy and bias using both incurred shrimp and fortified shrimp.—(*1*) *Incurred shrimp*.—For each matrix (raw head-on, raw head-off, and boiled shrimp), three lots of product known to contain sulfites were obtained. Three hundred grams of each lot were mixed, and three 50 g test portions were weighed out for the OMA method and five 2 g test portions for the Biofish method. Each 2 g blind-coded test portion was extracted and analyzed according to the Biofish method as detailed above (*see Sample Preparation*), and each 50 g blind-coded test portion was analyzed according to the OMA **990.28** (1, 2).

(*2*) *Fortified shrimp*.—The method developer laboratory obtained 2000 g raw head-off, raw head-on, and boiled shrimp with no sulfite added. Four sodium metabisulfite solutions were prepared such that three concentrations of fortified shrimp extending across each range of the Biofish method were prepared. The following method was used to fortify shrimp at each concentration: Submerge and swirl shrimp in metabisulfite solution and transfer to colander to drain without rinsing. From each fortified batch and from the unfortified matrix, three 50 g test portions for the OMA method were weighed out, and five 2 g test portions for the Biofish method were weighed out. The Biofish method was repeated on a second day using a second analyst.

(*3*) *Recovery experiment*.—Each matrix was examined sulfite-free and fortified at three concentrations (50, 100, and 250 mg/kg) with sodium metabisulfite using the following method: Fixed amounts of raw head-off, raw head-on, and boiled shrimp with no sulfite added were peeled, minced, and inoculated with the known concentration of sodium metabisulfite. The Biofish method was performed on blinded replicate 2 g test

Table 4. Selectivity results

		Raw shrimp				Boiled	
Compound, 100 ppm	Direct measure at 100 ppm	Head on	Head on rec., %	Head off	Head-off rec., %	Boiled shrimp	Boiled shrimp rec., %
Sodium sulfite	98	89	91	100	103	98	100
Sodium bisulfite	104	90	87	94	91	99	96
Sodium metabisulfite	98	91	93	93	95	101	104
Potassium metabisulfite	96	81	85	87	91	100	104
Formaldehyde-bisulfite adduct	103	87	84	89	86	94	91
Methionine	0	0	—[a]	0	—	0	—
Cysteine	0	0	—	0	—	0	—

[a] — = Not applicable.

portions and was repeated on a second day with a second analyst. Results were compared with the known fortification level.

(**b**) *Results*.—Accuracy, bias, and repeatability results are presented in Tables 5–10, and Figure 9–14 show the regression analysis for each matrix. Accuracy was assessed as a comparison of the Biofish results with the OMA results and ranged from 82 to 115% in all studied cases except for fortified raw shrimp head-on, in which the low level yielded an accuracy of 138%. It should be noted, however, that the low level in this case was below the claimed range of the method. The method bias was, in general, negative in both incurred and fortified high levels and slightly positive in incurred low levels. In the case of incurred boiled shrimp, the Biofish method had a slightly negative bias in all levels except for mid-level (100–150 ppm), in which the bias was positive. In the case of fortified shrimp head-on, the Biofish method had a positive bias at all levels. Repeatability was very good as shown by the low RSD_r values, demonstrating acceptable repeatability precision. Only three values were >10% RSD_r and <20%. Regression analyses showed a good correlation between the Biofish and OMA methods, with $R^2 > 0.99$ in all cases.

(*1*) *LOD and LOQ*.—LOD and LOQ were determined by first plotting S_r against the mean results and performing linear regression for each matrix and each quantification range. Next, LOD was calculated as

$$\mathrm{LOD} = \frac{\bar{X}_0 + 3.3(s_b)}{1 - 1.65m}$$

where $\bar{X}_0$ = the mean analytical value of the known negative matrix; S_b = the intercept of the plot; and m = the slope of the plot. LOQ is then estimated as LOQ = 3 × LOD.

These results are presented in Tables 11 and 12. The estimated LOQ value in the range 30–150 ppm was 9.97 mg/kg for raw shrimp head-off, 32.27 mg/kg for raw shrimp head-on, and 23.29 mg/kg for boiled shrimp. In the range 50–300 ppm, the estimated LOQ value was 20.10 mg/kg for raw shrimp head-off, 21.32 mg/kg for raw shrimp head-on, and 18.39 mg/kg for boiled shrimp. The LOQ for raw shrimp head-off, raw shrimp head-on, and boiled shrimp fish was validated at 30 mg/kg for quantification range 30–150 mg/kg and at 50 mg/kg for quantification range 50–300 mg/kg by spiking each matrix at

Table 5. Method developer results for incurred raw shrimp head-on

Level	Method (range, mg/kg)	N	Day/analyst	Result, mg/kg	Accuracy, %[a]	Bias Biofish[b]	Sr	RSD_r, %[c]
Low	OMA	3	Day 1	32	—[d]	—	1.2	3.6
	Biofish (30–150)	5	Day 1/analyst 1	34	109	2.7	0.9	2.6
			Day 2/analyst 2	35	109	2.9	1.5	4.4
Medium	OMA	3	Day 1	91	—	—	10.6	11.6
	Biofish (50–300)	5	Day 1/analyst 1	93	102	1.8	5.0	5.4
			Day 2/analyst 2	95	104	3.8	7.0	7.4
High	OMA	3	Day 1	197	—	—	3.1	1.6
	Biofish (50–300)	5	Day 1/analyst 1	175	89	–21.9	7.4	4.2
			Day 2/analyst 2	173	88	–23.7	11.6	6.7

[a] $(\mathrm{mean}_{\mathrm{Biofish}} / \mathrm{mean}_{\mathrm{OMA}}) \times 100$.

[b] $\mathrm{mean}_{\mathrm{Biofish}} - \mathrm{mean}_{\mathrm{OMA}})$.

[c] $(s_r / \mathrm{mean}_{\mathrm{cand}}) \times 100$.

[d] — = Not applicable.

Table 6. Method developer results for incurred raw shrimp head-off

Level	Method (range, mg/kg)	N	Day/analyst	Result, mg/kg	Accuracy, %[a]	Bias Biofish[b]	Sr	RSD_r, %[c]
Low	OMA	3	Day 1	23	—[d]	—	1.7	7.5
	Biofish(30–150)	5	Day 1/analyst 1	26	115	3.4	1.1	4.3
			Day 2/analyst 2	26	114	3.2	1.6	6.3
Medium	OMA	3	Day 1	91	—	—	6.4	7.0
	Biofish (50–300)	5	Day 1/analyst 1	98	108	7.3	4.4	4.5
			Day 2/analyst 2	97	107	5.9	3.0	3.1
High	OMA	3	Day 1	157	—	—	2.9	1.8
	Biofish (50–300)	5	Day 1/analyst 1	157	100	–0.5	5.3	3.4
			Day 2/analyst 2	153	97	–4.1	7.1	4.6

[a] $(\mathrm{mean}_{\mathrm{Biofish}} / \mathrm{mean}_{\mathrm{OMA}}) \times 100$.

[b] $\mathrm{mean}_{\mathrm{Biofish}} - \mathrm{mean}_{\mathrm{OMA}})$.

[c] $(s_r / \mathrm{mean}_{\mathrm{cand}}) \times 100$.

[d] — = Not applicable.

Table 7. Method developer results for incurred boiled shrimp

Level	Method (range, mg/kg)	N	Day/analyst	Result, mg/kg	Accuracy, %[a]	Bias biofish[b]	Sr	RSDr, %[c]
Low	OMA	3	Day 1	48	—[d]	—	1.5	3.2
	Biofish (30–150)	5	Day 1/analyst 1	44	92	–3.9	1.9	4.4
		5	Day 2/analyst 2	46	96	–1.9	2.3	5.0
Medium	OMA	3	Day 1	55	–	–	2.3	4.2
	Biofish (50–300)	5	Day 1/ analyst 1	58	106	3.3	2.2	3.9
		5	Day 2/analyst 2	54	98	–0.9	2.8	5.2
High	OMA	3	Day 1	289	—	—	2.0	0.7
	Biofish (50–300)	5	Day 1/analyst 1	254	88	–35.2	12.9	5.1
		5	Day 2/analyst 2	262	91	–26.8	8.3	3.2

[a] $(mean_{Biofish} / mean_{OMA}) \times 100$.

[b] $mean_{Biofish} – mean_{OMA})$.

[c] $(s_r / mean_{cand}) \times 100$.

[d] — = Not applicable.

Table 8. Method developer results for raw shrimp head-off fortified with sodium metabisulfite

Level	Method (range, mg/kg)	N	Day/analyst	Result, mg/kg	Accuracy, %[a]	Bias Biofish[b]	Sr	RSD_r, %[c]
Low	OMA	3	Day 1	28	—[d]	—	1.5	5.5
	Biofish (30–150)	5	Day 1/analyst 1	30	109	2.5	1.6	5.4
		5	Day 2/analyst 2	30	108	2.3	1.7	5.8
Medium 1	OMA	3	Day 1	53	—	—	4.0	7.7
	Biofish (30–150)	5	Day 1/analyst 1	57	109	4.5	2.6	4.5
		5	Day 2/analyst 2	55	104	2.1	2.6	4.7
Medium 2	OMA	3	Day 1	87	—	—	3.5	4.1
	Biofish (30–150)	5	Day 1/analyst 1	99	115	12.7	4.3	4.4
		5	Day 2/analyst 2	90	104	3.7	3.2	3.6
High	OMA	3	Day 1	340	—	—	3.0	0.9
	Biofish (50–300)	5	Day 1/analyst 1	292	86	–48.2	13.1	4.5
		5	Day2/analyst 2	280	82	–60.2	10.2	3.6

[a] $(mean_{Biofish} / mean_{OMA}) \times 100$.

[b] $mean_{Biofish} – mean_{OMA})$.

[c] $(s_r / mean_{cand}) \times 100$.

[d] — = Not applicable.

Table 9. Method developer results for raw shrimp head-on fortified with sodium metabisulfite

Level	Method (range, mg/kg)	N	Day/analyst	Result, mg/kg	Accuracy, %[a]	Bias Biofish[b]	Sr	RSD_r, %[c]
Low	OMA	3	Day 1	16	—[d]	—	3.1	18.7
	Biofish (30–150)	5	Day 1/analyst 1	23	138	6.3	1.7	7.4
			Day 2/analyst 2	21	130	4.9	1.6	7.8
Medium 1	OMA	3	Day 1	50	—	—	2.1	4.2
	Biofish (30–150)	5	Day 1/analyst 1	49	99	–0.5	2.3	4.6
			Day 2/analyst 2	53	108	3.7	2.4	4.5
Medium 2	OMA	3	Day 1	87	—	—	3.8	4.3
	Biofish (50–300)	5	Day 1/analyst 1	91	104	3.7	2.2	2.5
			Day 2/analyst 2	90	103	2.9	1.8	2.0
High	OMA	3	Day 1	138	—	—	17.3	12.6
	Biofish (50–300)	5	Day 1/analyst 1	150	109	12.2	6.2	4.1
			Day2/analyst 2	146	106	8.2	8.5	5.8

[a] $(mean_{Biofish} / mean_{OMA}) \times 100$.

[b] $mean_{Biofish} – mean_{OMA})$.

[c] $(s_r / mean_{cand}) \times 100$.

[d] — = Not applicable.

Table 10. Method developer results for boiled shrimp fortified with sodium metabisulfite

Level	Method (range, mg/kg)	N	Day/analyst	Result, mg/kg	Accuracy, %[a]	Bias Biofish[b]	Sr	RSD_r, %[c]
Low	OMA	3	Day 1	29	—[d]	—	2.9	9.8
	Biofish (30–150)	5	Day 1/analyst 1	30	102	0.7	2.1	6.9
			Day 2/analyst 2	31	106	1.7	1.5	4.9
Medium 1	OMA	3	Day 1	53	—	—	2.5	4.7
	Biofish (30–150)	5	Day 1/analyst 1	53	100	0	3.0	5.6
			Day 2/analyst 2	52	98	–1.0	3.6	6.9
Medium 2	OMA	3	Day 1	125	—	—	3.1	2.5
	Biofish (50–300)	5	Day 1/analyst 1	120	96	–4.7	3.7	3.1
			Day 2/analyst 2	120	96	–4.5	1.8	4.5
Low	OMA	3	Day 1	294	—	—	3.0	1.0
	Biofish (50–300)	5	Day 1/analyst 1	276	94	–17.7	8.6	3.1
			Day2/analyst 2	283	96	–10.8	7.9	2.8

[a] $(mean_{Biofish} / mean_{OMA}) \times 100$.

[b] $mean_{Biofish} - mean_{OMA})$.

[c] $(s_r / mean_{cand}) \times 100$.

[d] — = Not applicable.

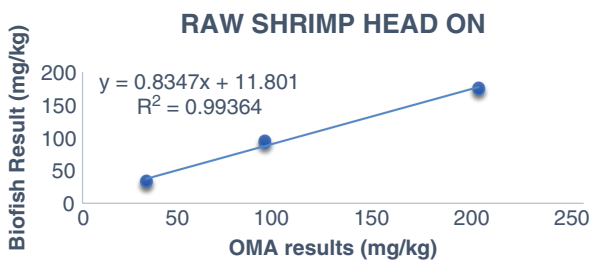

Figure 9. Graph of mean Biofish results versus OMA results for incurred raw shrimp head-on.

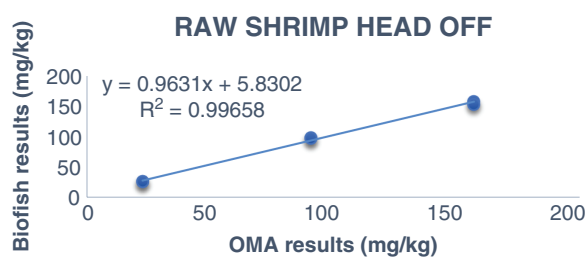

Figure 10. Graph of mean Biofish results versus OMA results for incurred raw shrimp head-off.

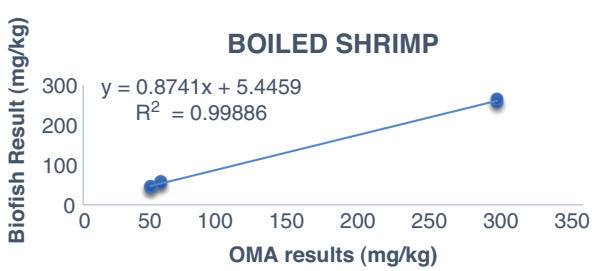

Figure 11. Graph of mean Biofish results versus OMA results for incurred boiled shrimp.

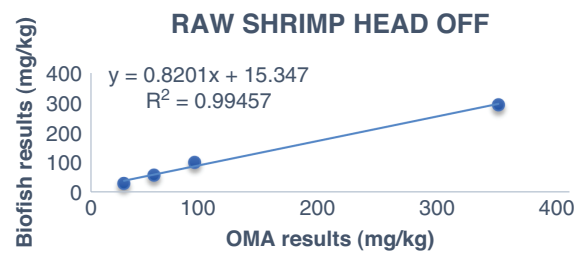

Figure 12. Graph of mean Biofish results versus OMA results for raw shrimp head-off fortified with sodium metabisulfite.

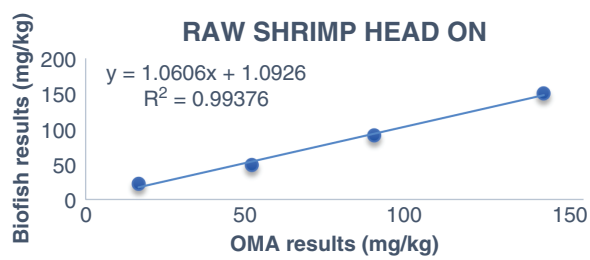

Figure 13. Graph of mean Biofish results versus OMA results for raw shrimp head-on fortified with sodium metabisulfite.

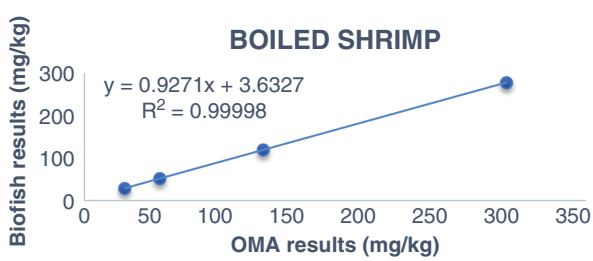

Figure 14. Graph of mean Biofish results versus OMA results for boiled shrimp fortified with sodium metabisulfite.

Table 11. Method developer estimation of LOD and LOQ in range 30–150 mg/kg

	Raw shrimp		Boiled shrimp
	Head off	Head on	
X_0	5.0	6.5	5.0
S_b	–0.4536	1.2593	0.8082
m	0.0651	0.0146	0.0209
LOD	3.32	10.76	7.76
LOQ = 3 × LOD	9.97	32.27	23.29

Table 12. Method developer estimation of LOD and LOQ in range 50–300 mg/kg

	Raw shrimp		Boiled shrimp
	Head off	Head on	
X_0	5.0	6.5	5.0
S_b	0.4773	0.171	0.3267
m	0.0442	0.0419	0.0285
LOD	6.70	7.11	6.13
LOQ = 3 × LOD	20.10	21.32	18.39

Table 13. Method developer validation of LOQ by matrix

Matrix	Sulfite concn level, mg/kg	Mean result, mg/kg	S_r	RSD_r
Raw shrimp head-on	30	17	2.63	15.1
Raw shrimp head-off	30	28	1.58	5.7
Boiled shrimp	30	30	1.70	5.6
Raw shrimp head-on	50	31	1.89	6.2
Raw shrimp head-off	50	37	1.26	3.4
Boiled shrimp	50	45	1.35	3.0

those levels with sodium metabisulfite and testing 10 replicate test portions. These results are presented in Table 13. The RSD_r values were all below 16%, with only one value above 10%. Thus, the LOQ values were validated.

(*2*) *Recovery*.—Results of the recovery experiment are presented in Table 14. All recovery values ranged between 80 and 110%, except in the case of raw shrimp head-on at 50 mg/kg on day 1/analyst 1, in which the obtained result was 78% for recovery. However, the same sample measured by analyst 2 on day 2 showed a result of 86% for recovery.

Lot-to-Lot Consistency and Stability Study

(**a**) *Methodology*.—The Biofish-300 SUL method consists of a calibration kit, a measurement kit, and the Biotest electrode. Lot-to-lot data were collected for three lots of each kit. Stability was evaluated over 6 months for the calibration kit and measurement kit. Four tubes are supplied in the calibration kit and eight or five tubes in each measurement kit, so it was necessary to use a new tube of each at each of the six time points. At the 1 month point, two additional lots were tested to

Table 14. Recovery of sodium metabisulfite by matrix

Matrix	Known sodium metabisulfite concn, mg/kg	Day/analyst	Biofish results, mg/kg	Avg., mg/kg	Rec., %
Raw shrimp head-off	0	Day 1/analyst 1	6	2.2	
			0		
			0		
			0		
			5		
		Day 2/analyst 2	0	0.0	
			0		
			0		
			0		
			0		
	50	Day 1/analyst 1	41	40	80
			42		
			37		
			40		
			40		
		Day 2/analyst 2	39	41	82
			42		
			42		
			41		
			40		
	100	Day 1/analyst 1	81	83	83
			83		
			84		
			83		
			82		
		Day 2/analyst 2	81	83	83
			82		
			83		
			83		
			84		
	250	Day 1/analyst 1	233	237	95
			237		
			237		
			239		
			241		
		Day 2/analyst 2	233	236	94
			240		
			235		
			236		
			235		
Raw shrimp head-on	0	Day 1/analyst 1	0	2.0	
			5		
			0		
			0		
			5		
		Day 2/analyst 2	5	1.0	
			0		
			0		
			0		
			0		

Table 14. (*continued*)

Matrix	Known sodium metabisulfite concn, mg/kg	Day/analyst	Biofish results, mg/kg	Avg., mg/kg	Rec., %
	50	Day 1/analyst 1	41	39	78
			38		
			39		
			37 39		
		Day 2/analyst 2	40	43	86
			43		
			42		
			48		
			42		
	100	Day 1/analyst 1	82	83	83
			82		
			83		
			83		
			85		
		Day 2/analyst 2	86	83	83
			85		
			80		
			81		
			83		
	250	Day 1/analyst 1	220	222	89
			223		
			221		
			225		
			222		
		Day 2/analyst 2	217	216	86
			218		
			215		
			215		
			216		
Boiled shrimp	0	Day 1/analyst 1	0	0.0	
			0		
			0		
			0		
			0		
		Day 2/analyst 2	0	0.0	
			0		
			0		
			0		
			0		
	50	Day 1/analyst 1	47	46	92
			45		
			45		
			46		
			46		
		Day 2/analyst 2	44	44	89
			45		
			43		
			46		
			44		
	100	Day 1/analyst 1	93	91	91
			91		
			86		
			94		
			92		
		Day 2/analyst 2	88	87	87
			87		
			87		
			86		
			87		
	250	Day 1/analyst 1	253	254	102
			251		
			261		
			254		
			253		
		Day 2/analyst 2	252	250	100
			247		
			254		
			245		
			252		

evaluate the lot-to-lot consistency. The Biotest stability was examined over a 30 day period in a separate experiment. One lot of electrodes was chosen and tested with one kit each of the calibration and measurement kits over the course of 30 days, using a fresh electrode for each time point (0, 2, 5, 10, 20, and 30 days after manufacture). One time point was chosen to test three different lots of electrodes. A single kit for each of KITCFSUL, KITMFSUL1, and KITMFSUL2, using a fresh tube for each time point, was used. In both experiments, two replicates of shrimp with 50–100 ppm sulfite per time point or lot were analyzed. Incurred shrimp head-off with 50–100 ppm of sulfite was used.

(**b**) *Results*.—Results for the lot-to-lot testing and the 6-month stability for the calibration and measurement kits are presented in Table 15. Data were analyzed by generalized linear model (GLM) analysis using SPSS statistics version 23. GLM analysis showed that there were no significant differences between Biotest electrode lots evaluated at month 1, nor between the time points over the 6-month period for the calibration and measurement kits. A plot of the stability data indicated no trend in the results over time (Figure 15).

The results of the 30-day stability of the Biotest electrode are shown in Table 16. The GLM results obtained showed a significant difference between results on day 5 and day 30. It is observed that the day 5 results were slightly lower than the rest of the evaluated days, and the day 30 results were slightly higher than the rest of evaluated days. However, GLM analysis showed that there were no significant differences between day 5 and day 30 when compared with the rest of the evaluated days. A plot of the stability data indicated no trend over time (Figure 16), nor were there significant differences between the different lots of biotest evaluated at day 2 of the study.

Table 15. Lot-to-lot results and 6 month stability for the calibration and measurement kits

Study time, months	Calibration kits, lots	Measurement kit, lots	Biotest (different lots every month of study)	Biofish results in shrimp with 50–100 ppm sulfites	Avg.	S_r	RSD_r
0	C1	M1	B1	69	68.0	1.41	2.08
				67			
1	C1	M1	B2	63	63.5	0.71	1.11
				64			
	C2	M2		67	68.5	2.12	3.10
				70			
	C3	M3		68	68.5	0.71	1.03
				69			
2	C1	M1	B3	74	72.0	2.83	3.93
				70			
3	C1	M1	B4	73	71.0	2.83	3.98
				69			
4	C1	M1	B5	68	67.0	1.41	2.11
				66			
6	C1	M1	B6	67	68.0	1.41	2.08
				69			

Table 16. Lot-to-lot results and 30 day stability for the Biotest electrode

Study time, days	Calibration kit (different lots every day)	Measurement kit (different lots every day)	Biotest, lots	Biofish results	Avg.	S_r	RSD_r
0	C1	M1	B1	67	68.0	1.41	2.08
				69			
2	C1	M1	B1 B2 B3	70	70.0	0.00	0
				70			
				68	68.5	0.71	1.03
				69			
				67	66.5	0.71	1.06
				66			
5	C1	M1	B1	64	64.5	0.71	1.10
				65			
10	C1	M1	B1	67	69.0	2.83	4.10
				71			
20	C1	M1	B1	70	68.5	2.12	3.10
				67			
30	C1	M1	B1	73	72.5	0.71	0.98
				72			

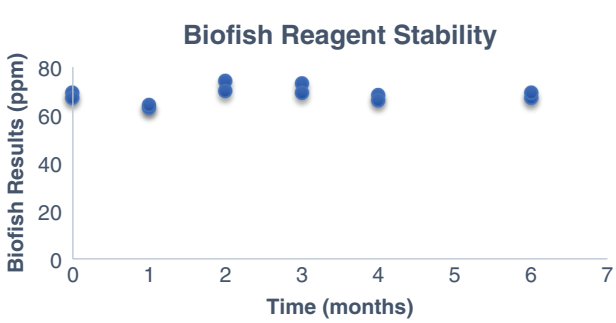

Figure 15. 6 month stability plot.

Robustness

(**a**) *Methodology*.—Three method parameters were varied, including extraction volume, ultraturrax time, and extract storage time before analysis. The parameter values are shown in Table 17, and Table 18 shows the factorial design employed. Treatment combination 9 uses the normal method parameter values. Treatment combinations 1–8 use either the high or low values for each parameter. For each treatment combination, two replicates of incurred raw shrimp head-off with 50–100 ppm sulfite (measured as SO_2) were analyzed.

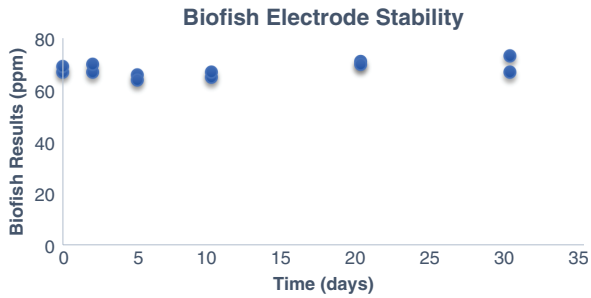

Figure 16. 30 day stability plot.

Table 17. Robustness parameters: raw shrimp head-off

Robustness parameters	Values		
	1	2	3
Extraction volume, mL	16	18	20
Ultraturrax time, sec	10	20	30
Extract storage before analysis	None	2 h at 25°C	4 h at 25°C

(**b**) *Results*.—The results of the duplicate analyses are shown in Table 18. The factorial design was translated into the Youden design (1) in Table 19, and the Youden test was applied (Table 20).

According to the results obtained applying the Youden test, significant differences were observed in all the studied cases. The evaluated variations adversely affected the analytical results according to the Youden test. It was observed that the method deviation in the case of raw shrimp head-off with 50–100 ppm sulfite (measured as SO_2) was low, and for this reason, the acceptance criteria seems to be too strict. The acceptance criteria are from Commission Directive No. 2002/357/EC concerning the performance of analytical methods and the interpretation of results (4).

To confirm the results obtained by the Youden test, a GLM was applied to determine whether any parameter change affects the results. In this case, significant differences were observed in the results when extraction volume, ultraturrax time, and time of extract storage before analysis were varied. Post hoc results confirmed that significant differences were obtained between 16 and 18 mL and between 16 and 20 mL of extraction volume. Significant differences were also found between 10 and 30 s of time and between 0 and 2 h of extract storage before analysis. In the case of time of extract storage before analysis, the difference was attributed to one of the replicates at 2 h of storage, which showed a high level

Table 18. Factorial design and results of robustness testing

Raw shrimp head-off with 50–100 ppm sulfite	Treatment combination								
	1	2	3	4	5	6	7	8	9
Extraction volume, mL	16	16	16	16	20	20	20	20	18
Ultraturrax time, s	10	10	30	30	10	10	30	30	20
Extract storage before analysis	None	4 h at 25°C	None	4 h at 25°C	None	4 h at 25°C	None	4 h at 25°C	2 h at 25°C
	100	114	101	116	84	102	88	98	111
Biofish results, mg/kg	99	115	102	110	82	95	92	96	102

Table 19. Youden factorial design

F factor	Value of *F* factor	Determination combination								
		1	2	3	4	5	6	7	8	9
Extraction volume, mL	A = 16	A	A	A	A	a	a	a	a	a´
	a = 20									
	a´ = 18									
Ultraturrax time, s	B = 10	B	B	b	b	B	B	b	b	b´
	b = 30									
	b´ = 20									
Extract storage before analysis, h	C = none	C	c´	C	c´	C	c´	C	c´	c
	c = 2									
	c´ = 4									
Result (avg. data), mg/kg		99.5	114.5	101.5	113	83	98.5	90	97	106.5
Nominal, %[a]		93.4	107.5	95.3	106.1	77.9	92.5	84.5	91.1	NA[b]

[a] Combination 9.
[b] NA = Not applicable.

Table 20. Youden test results

Variables comparison (X–x)	Avg. differences (D_i)	SD of the differences	SD of the method (raw shrimp head-off, 106 mg/kg)	Acceptance criterion	Compliance[a]
Δ A, a[b,c]	15.00	7.50			No
Δ A, a´[d]	6.63	3.31			No
Δ a, a´	14.38	7.19			No
Δ B, b[e,f]	11.31	5.66			No
Δ B, b´[g]	11.63	5.81			No
Δ b, b´	9.38	4.69			No
Δ C, c[h,i]	13.00	6.50			No
Δ C, c´[j]	13.63	6.81			No
Δ c, c´	8.00	4.00	2.8	$SD_i \leq SD$[k]	No

[a] Indicates whether results are in compliance with acceptance criterion.

[b] A = 16.

[c] a = 18.

[d] a` = 20.

[e] B = 10.

[f] b = 30.

[g] b` = 20.

[h] C = 0.

[i] c = 2.

[j] c` = 4.

[k] SD_i = SD of the differences.

of sulfite (111 ppm). If this replicate is not considered, no significant differences are found for the time of storage before analysis.

Reference Material

Reference material GSCCA-1/2016 (5) of peeled shrimp was analyzed during four different days and percent of recovery of the Biofish results was calculated.

Results of the recovery experiment are presented in Table 21. All recovery values ranged between 87 and 91%.

Independent Laboratory Study

The independent validation study was conducted by Eurofins Nutrition Analysis Center (USA) under the guidance of the AOAC-RI.

(**a**) *Methodology*.—The Biofish-300 SULFITE method kit (both 30–150 and 50–300 mg/kg modes) was validated against the OMA **990.28** for sulfites in foods, using samples of raw, headless shrimp from a local grocery store spiked with sodium metabisulfite. Each sample was peeled, minced, and split into 200 g portions, and different concentrations of a sodium bisulfite solution were added by spraying the shrimp with a small volume of spiking solution before the whole sample was blended for 30 s. Sample 1 had no spike. Sample 2 received 10 mL of 1.5846 mg/mL sodium metabisulfite. Sample 3 received 20 mL of 1.5846 mg/mL sodium metabisulfite. Sample 4 received 30 mL of 1.5846 mg/mL sodium metabisulfite. Sample 5 received 30 mL of 3.1367 mg/mL sodium metabisulfite. For analysis, three 50 g portions and five 2 g portions were weighed and tested by Monier-Williams method and the Biofish System respectively. Samples 2, 3, and 4 were spiked and tested on day 1, and samples 1 and 5 were spiked and tested on day 2.

Table 21. Results of reference material GSCCA 1/2016

Matrix	Ref. value GSCCA 1/2016, mg/kg SO_2	Days of analyses	Biofish results	Avg.	Rec., %
Peeled shrimp	116	1	98	106	91
			114		
			105		
			107		
		2	93	101	87
			106		
			103		
		3	100	108	93
			112		
			107		
			112		
		4	94	101	87
			107		
			101		

The equipment used was a Biofish-300, serial No. 17012 (Biolan Microbiosensores); a sulfite biotest for 50 analysis (ID SUL50626437; Biolan Microbiosensores); a generic kitchen blender; and a Cole-Parmer Labgen 7 Homogenizer, up to 33k rpm, Serial No. C12520320. The reagent list included a calibration standard (KITCFSUL; Lot No. CFSUL280317; Biolan Microbiosensores), a sulfite extraction reagent (SEFSUL; Lot No. SEFSUL2200117/1; Biolan Microbiosensores), and a sulfite measurement reagent (KITMFSUL2; Lot No. MFSUL2261216/2; Biolan Microbiosensores).

(**b**) *Results*.—Table 22 summarizes the results obtained by the independent laboratory. The Biofish system showed linearity and consistency across the two detection ranges. Accuracy, assessed as a comparison of the Biofish results with the OMA results, ranged from 103 to 160%. However, recovery percentage shows better theoretical recovery in the Biofish method than the OMA method, which could explain the high accuracy values of the Biofish compared with the OMA method. In fact, recoveries ranged between 61 and 76% in the OMA method, while in the Biofish method, they were between 78 and 98% in 30–150 mg/kg range and between 81 and 88% in 50–300 mg/kg range. The method bias was positive in both detection ranges, generally with values between 10 and 35; however, at high levels, the Biofish system compared better with the Monier-Williams method. These values agree with an observed sulfite recovery of 61–76% for the OMA method. Repeatability was excellent as demonstrated by the low RSD_r values, which ranged from 0.9 to 7.9 in all tested samples.

Regression analyses showed a very good correlation between the Biofish and OMA methods. The R^2 value was >0.99 for both detection ranges (Figure 17 and 18).

Table 22. Results obtained by the independent laboratory (all sulfite results expressed in mg/kg)

		Experiment date: 9/21/2017				Experiment date: 9/22/2017	
		Sample 2	Sample 3	Sample 4		Sample 1	Sample 5
Portion 1		32	74	112		<10	237
Portion 2		35	73	99		<10	238
Portion 3		33	76	108		<10	244
Mean	Monier-Williams	33	74	106	Monier-Williams	—[a]	240
RSD_r, %		6.1	1.8	6.3		—	1.6
Theoretical level SO_2, mg/kg		54	107	160		0	317
Rec., %[b]		61	69	66		—	76
Portion 1		50	97	145		0	253
Portion 2		55	101	145		0	242
Portion 3		55	105	144		0	251
Portion 4		54	105	142		0	249
Portion 5		52	103	141		0	245
Mean	Biofish[c]	53	102	143	Biofish[d]	0	248
s_r		2.2	3.3	1.8		—	4.5
RSD_r, %		4.1	3.3	1.3		—	1.8
Accuracy, %[e]		160	138	135		—	103
Bias		+20	+28	+37		—	+8
Theoretical level SO_2, mg/kg		54	107	160		0	317
Rec., %[f]		98	95	89		0	78
Portion 1		40	93	140		0	273
Portion 2		46	95	141		0	262
Portion 3		44	92	138		0	265
Portion 4		49	91	140		0	262
Portion 5	Biofish[g]	42	98	141	Biofish[h]	0	260
Mean		44	94	140		0	264
s_r		3.5	2.8	1.2		—	5.1
RSD_r, %		7.9	3.0	0.9		—	1.9
Accuracy, %[e]		133	126	132		—	110
Bias		+11	+20	+ 34		—	+25

[a] — = Not applicable.

[b] ($Mean_{MW}$ / theoretical level) × 100.

[c] 30–150 mg/kg range; R = 0.9999; m = –219486.84; b = –1.53.

[d] 30–150 mg/kg range; R = 1; m = –221557.00; b = –0.01.

[e] ($mean_{Biofish}$ / $mean_{OMA}$) × 100.

[f] ($Mean_{Biofish}$ / theoretical level) × 100.

[g] 50–300 mg/kg range; R = 0.9999; m = –229581.62; b = –1.27.

[h] 50–300 mg/kg range; R = 0.9999; m = –220264.96; b = –1.56.

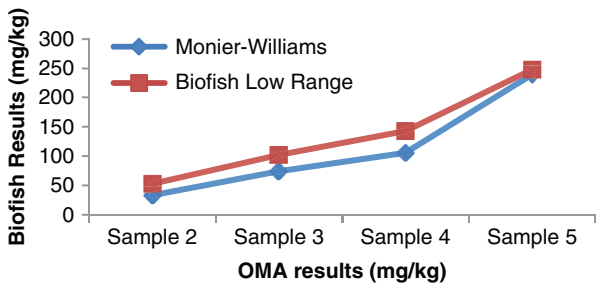

Figure 17. Correlation plot of Biofish method (low-range) versus Monier-Williams method. Linear regression equation: $Result_{Biofish}$ = $Result_{OMA}$ × 0.9203 + 32.4114; R = 0.9928.

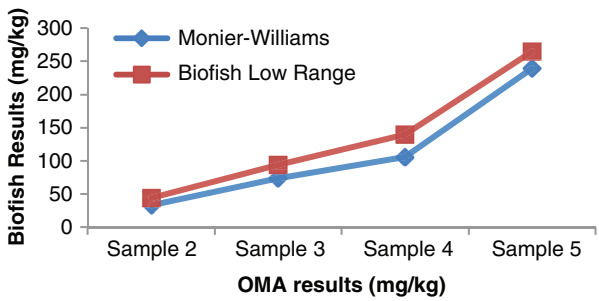

Figure 18. Correlation plot of Biofish method (high-range) versus Monier-Williams method. Linear regression equation: $Result_{Biofish}$ = $Result_{OMA}$ × 1.0515 + 16.4368; R = 0.9961.

Table 23. LOD and LOQ estimates from the independent lab data

	X_0	S_b	m	LOD	LOQ
Low-range method, 30–150 mg/kg	0	1.53	0.01	5.13	15.39
High-range method, 50–300 mg/kg	0	1.98	0.01	6.64	19.92

LOD and LOQ were estimated as in the method developer study. The estimated values are presented in Table 23. The tested matrix yielded estimated LOQ values lower than 20 mg/kg for both method ranges.

Discussion

The aim of the present work was to receive PTM certification for the Biofish-300 SUL method for the detection and quantification of sulfites in shrimp. This technology is highly specific and selective and allows quantification of sulfites in raw shrimp head-on, raw shrimp head-off, and boiled shrimp. Sulfite in all the studied matrixes was extracted in an extraction solution in a short time (15–20 s).

The linearity and matrix studies demonstrated linear dose responses from 0 (nondetect) to 300 mg/kg sulfite. As a whole, accuracy, recovery, and bias within range results indicate the kit provides accurate and precise sulfite quantification for all the evaluated matrixes, confirming that sample preparation and assay procedures produce acceptable results.

The analyses of reference material have shown that the results obtained for sulfite were adequate in peeled shrimp.

Data produced in the lot-to-lot consistency and stability study show consistent results for calibration and measurement kit components during 6 months in dry storage conditions and during 30 days for the Biotest electrodes.

The robustness study showed that variation of the extract volume and ultraturrax time produced unacceptable results. The package insert defines the correct protocols; the procedure must be performed according to these instructions to avoid mistakes.

The results obtained in the study were similar to those performed by the independent laboratory. The analysis performed by the independent laboratory with fortified shrimp showed very good accuracy and precision. The LOQ was lower than 20 ppm for both detection ranges of the method.

From the independent laboratory point of view, the Biofish system was fast and convenient to carry out and demonstrated precision, excellent linearity, and consistency across the two detection ranges.

Conclusions

In summary, the Biofish-300 SUL method is precise and accurate in all three evaluated shrimp matrixes. The LOQ was established at 30 mg/kg for the evaluated matrixes. The method is selective, robust, consistent, and stable in most of the studied circumstances.

References

(1) Youden, W.J., & Steiner, E.H. (1975) *Statistical Manual of the Association of Official Analytical Chemists, Part 1*, AOAC, Washington, DC, p. 35
(2) *Official Methods of Analysis* (1995) 16th Ed., AOAC INTERNATIONAL, Gaithersburg, MD, Method **990.28**
(3) Commission Regulation (EU) No. 1129/2011 (2011) *Off. J. Eur. Comm.* **L295**, 1–177
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