Headspace Solid-Phase Microextraction: Validation of the Method and Determination of Allyl Isothiocyanate Persistence in Cowpea Beans

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ABSTRACT: Essential oils are widely recognized as an efficient and safe alternative for controlling pests in foods. However, a few studies have determined the persistence of these compounds in stored grains. The present study optimized and validated a fast and effective method for extraction and quantification of allyl isothiocyanate (AITC—the main component of mustard essential oil) residue in cowpea beans. It also investigated the persistence of this substance in the grains. The proposed method employs headspace solid-phase microextraction (HS-SPME) and gas chromatography with a flame ionization detector (GC/FID). For optimizing it, a central composite design (CCD) was used, where the best conditions for the extraction of the AITC were achieved using 15 min fiber exposure at 30 °C. The performance of the method was assessed by studying selectivity, linearity, limits of detection (LOD) and quantification (LOQ), precision, and accuracy. The LOD and LOQ for AITC were 0.11 and 0.33 μg kg⁻¹, respectively. The determination coefficient ($R^2$) was above 0.99. The relative recovery rate ranged from 108.2 to 114.8%, with an interday coefficient of variation below 9%. After 36 h, no residue was detected in the samples, demonstrating that the AITC has low persistence and can be safely used as a bioinsecticide for grains.

INTRODUCTION

Cowpea beans [Vigna unguiculata (L.) Walp.] is one of the five most cultivated legumes in the tropical belt due to both its cultivation versatility and tolerance to drought periods and hot temperatures.₁,₂ Additionally, it is a food source rich in proteins, minerals, vitamins, and fibers, which can be cooked in little time. These factors favorably contribute to its impact on the daily diet of tropical and subtropical populations in Africa, Asia, Europe, and America.³ To preserve the nutritional value of cowpea beans and assure its viability for human consumption, the grains must be appropriately stored after harvest. Even so, throughout storage, cowpea beans face both quantitative and qualitative losses owing to the attack of the beetle Callosobruchus maculatus (Fabr., 1775) (Coleoptera: Chrysomelidae).⁴

Phosphine is the only insecticide currently allowed in Brazil for controlling C. maculatus in cowpea beans.⁵ However, its use poses risks for the environment and both animal and human health.⁶ It may also lead to the development of more resistant insect populations.⁷⁻⁹ On that account, efforts have been made to find plant-based products with characteristics and purposes similar to conventional insecticides.¹⁰ Bioinsecticides are acknowledged as safer than their traditional counterparts, with the advantage of not being connected to a resistance increase in the targeted plagues, nor presenting a risk to the environment or human and animal health.¹¹,¹²

Allyl isothiocyanate (AITC) is a volatile substance that can be found on several cruciferous vegetables of the genus Brassica, common in human diet, and known for its toxicity and repellent activity to various storage pests,¹³⁻¹⁶ including the cowpea weevil. In fact, human exposure to AITC is usual and quite frequent, and the compound is considered safe to both the environment and human health.¹⁷ Therefore, acceptable levels of the said exposure are yet to be established appropriately.¹⁸ Consequently, the evaluation of the persistence of insecticides is essential to guarantee food safety, help determine the withdrawal period, and also set the usage criteria.
for these agrochemicals. Some researchers have employed solid-phase microextraction (SPME) to determine phosphine traces (PH₃) in stored grains. However, no method has been reported to identify residues of AITC.

The techniques used to determine pesticide residues in complex samples have evolved significantly in terms of extraction and purification so as to minimize the utilization of solvents and reagents and optimize the cleaning process. Gas chromatography (GC) is prominent among the modern analytical methods of quantification of pesticides in different matrices. Through this technique, the substances are separated, identified, and quantified. Nonetheless, previous extraction and concentration steps are still required.

For extraction, solid-phase microextraction (SPME) is currently one of the most used techniques, especially for multisidue extraction of organic compounds. Past studies have proved the efficiency of this method for obtaining pyrethroids and organochlorines and have also shown the possibility of simultaneously extracting pesticides from different classes.

Headspace solid-phase microextraction (HS-SPME) has many advantages over other methods, as it reduces, or even eliminates, the need for organic solvents. Besides, it allows the simultaneous extraction and concentration of the analyte.

Due to negligence and the consequent exposition of people to agrochemicals, there is an increasing demand for pesticide-free food. This fact has instigated researchers to monitor and evaluate the risks involved in consuming contaminated foodstuff. In general, AITC has many attributes desirable in an insecticide, but further studies are still required to clarify the mechanisms of action and persistence. For that reason, this work aimed at optimizing and validating a method using headspace solid-phase microextraction (HS-SPME) and gas chromatography with a flame ionization detector (GC/FID) to determine the residue of AITC and its persistence in cowpea beans treated via fumigation.

RESULTS AND DISCUSSION

Chromatographic Analysis. The optimized chromatographic conditions allowed us to separate the intended analyte (AITC), within a running time of 6 min. In the chromatogram, AITC showed a double peak due to its conversion into isomers during the injection into the device. Therefore, it was necessary to add up the area of both isomer peaks to attain the value corresponding to the total AITC. Figure 1 represents the chromatogram obtained from cowpea beans fortified with AITC at the concentration of 22.42 μL kg⁻¹—the CL₉₅ used for controlling C. maculatus.

Preparation of Standard Solutions. First, preliminary tests were performed to select the organic solvents with no toxicity to adult insects of C. maculatus. The preselected solvents that did not cause mortality in C. maculatus insects (acetonitrile, ethanol, octanol, and toluene) were used in the as-prepared AITC solutions. Next, bean samples were fortified and subjected to extraction and analysis. The efficiency of the extraction, according to the solvent employed, complied with the following order: ethanol > octanol > acetonitrile > toluene (Figure 2). In this case, there was a significant difference among the solvents tested (F = 382.03, d.f.ₐₚₑₓₜ = 4, P < 0.0001), with ethanol displaying the largest chromatographic area and well-defined peaks, which is remarkably interesting, as ethanol is a low-cost, easily accessible reagent. Despite also presenting a good chromatographic result, octanol produced peaks that overlapped those of AITC. All assays were performed in triplicate. Thus, all subsequent steps of this work utilized ethanol for preparing the standard AITC solution.

Sample Preparation for Optimization Studies. To evaluate the influence of the fortification time of beans on the extraction of AITC, the times of 5, 10, and 15 min were tested. The times tested did not differ at a 5% probability level, according to the F test (F = 0.0522, d.f.ₐₚₑₓₜ = 3, P = 0.9499). Therefore, the shortest fortification time (5 min) was chosen to proceed with the method.

Testing was conducted to verify whether the flask condition (open or closed) during the fortification process would affect the chromatographic behavior of AITC. The AITC extractions were carried out at 40 °C for fiber exposition times of 8, 14, and 20 min. The combinations tested with flasks either with or without the cap and different extraction times varied significantly (F = 55.63, d.f.ₐₚₑₓₜ = 7, P < 0.0001). Except for the time of 8 min, the assays in which the flasks remained open during fortification produced responses with larger chromatographic areas. Regarding the duration of extraction, the best chromatographic areas were attained at 14 and 20 min, with no statistical difference between these two times.

Optimization Procedure. The intended optimization of the method was tackled in two ways: (i) univariate optimization of the fiber and (ii) multivariate optimization of both time and temperature of extraction. The most suitable

Figure 1. Chromatogram obtained after headspace microextraction in cowpea beans fortified with AITC (retention time = 3 min) at the concentration of 22.42 μL kg⁻¹.

Figure 2. Comparison among different solvents used for preparing the analytical standard AITC solution. Sample mass: 5 g; extraction: 40 °C for 10 min; DVB/CAR/PDMS fiber.
Fiber Coating Choosing. Choosing the adequate fiber is essential for establishing a sensitive method for solid-phase microextraction, and it strongly depends on the chemical nature of the compound of interest. In this study, the determination of the fiber for extracting AITC was based on the area of the chromatographic response of each one of the fibers exposed to the compound at 40 °C, considering the extraction time of 10 min (Figure 3). The behavior of the three fibers tested diverged significantly ($F = 653.45$, d.f. error = 6, $P < 0.0001$). The 50/30 μm DVB/CAR/PDMS StableFlex fiber differed from the 100 μm PDMS fused-silica and the 65 μm PDMS/DVB fused-silica ones, as it showed the largest chromatographic area as a response, which indicates the best AITC extraction.

Polarity, molecular weight, and the size of the analyte of interest are the criteria that must be pondered before choosing the most suitable fiber for applying the HS-SPME technique.25 Fibers coated with PDMS are generally better, as in the long term, they present stability and good performance during the processes of chromatography, extraction, and recovery of the analyte. Besides, these fibers are durable and usually hold satisfactory functioning for up to 100 analytical cycles. PDMS fibers are not polar; therefore, they are recommended by manufacturers for the extraction of apolar analytes, such as aromatic and volatile compounds from essential oils. In turn, mixed fibers, with a coating containing DVB or CAR, have an increased retention capacity due to a mutually potentializing effect on the extraction and distribution of the stationary phase. Finally, the triphasic DVB/CAR/PDMS fiber has a PDMS-DVB layer over a CAR-PDMS one. Its use is advised for the extraction of volatile and semivolatile compounds from food, as it provides high recovery rates of analytes with different structures and polarities.28

AITC is an extremely volatile, colorless to pale yellow liquid, slightly soluble in water, but with good solubility in most organic solvents ($\log k_{\text{ow}} = 2.15$). The extraction by the mixed coatings DVB/CAR/PDMS considers the properties of each constituent polymeric coating, which makes this coating ideal for extracting a wide range of analyte polarities (from C2 to C20).29 In addition, the micropores present in the carboxen polymer must have helped in the extraction, since this polymer has a strong affinity for relatively small sulfur-containing compounds, as is the case of AITC (CH$_2$=CHCH$_2$N=C=S).

Multivariate Analysis of Time and Temperature of Extraction. Some factors might influence the efficiency of headspace solid-phase microextraction, including the choice of the ideal fiber exposure time and water bath temperature. To better assess the most suitable settings regarding these aspects, a central composite design (CCD) with a $2^3$ factorial scheme and six central points was applied. The value of the area corresponding to AITC after a GC/FID analysis was used to build a Pareto chart (Figure 4a), which allowed us to evaluate the significance of the variables and their interactions through the length of the bars. Thus, the best AITC extraction condition was attained at 30 °C and 15 min, in which the GC/FID analysis revealed the largest chromatographic area. The linear regression analysis by response surface presented a linear behavior for both parameters ($R^2 = 0.87$). It also showed that the extraction time had a positive impact on the chromatographic response. In contrast, the temperature negatively affected the size of the area corresponding to AITC—both cases at a 5% probability level (Figure 4b).

The headspace solid-phase microextraction method involves a multiphase equilibrium process. In general, time and temperature of extraction are among the most important aspects that affect this technique, which makes them the object of investigations and optimization.21 The equilibrium time in the headspace is related to the extraction temperature, fiber coating thickness, and diffusion coefficient of the analytes.30 It can be observed in Figure 5 that with increasing temperature at
any extraction time there is a decrease in responses, indicating that higher temperatures impair the compound sorption process, favoring its desorption from the fiber. Hence, the extraction time must be enough to achieve the equilibrium condition, after which the quantity of analyte extracted will be constant.31

Validation of the Optimized Method. Method Selectivity. The optimized method was applied to pesticide-free cowpea bean samples to evaluate its selectivity. Subsequently, those samples were fortified with AITC and subjected again to the processes of extraction and analysis. The resulting chromatograms (Figure 6a and 6b) did not exhibit any interferent overlapping the analyte of interest. Thus, as the pesticide-free cowpea bean sample did not show any response of other substances at the same time as the compound tested, it can be said that the method is selective.

Limit of Detection (LOD) and Limit of Quantification (LOQ). LOD is the lowest analyte concentration traceable in a sample by any analytical method. LOQ, in turn, represents the lowest analyte concentration that can be quantified at an acceptable level of precision, while still complying with the criteria of repeatability and precision.32,33 The LOD and LOQ for AITC were determined by the signal/noise ratio, which in this research presented concentrations equal to 0.11 and 0.33 μg kg⁻¹, respectively. Currently, AITC is not registered for grain fumigation by any Brazilian regulatory agency, so there is no maximum residue limit (MRL) for this compound in stored products. Nazareth et al.,34 while studying the efficacy of AITC fumes for inhibiting fungi in maize, also validated a methodology for quantification of AITC grains. The authors carried out extractions with ethanol and ultrasound, as well as analyses via high-performance liquid chromatography (HPLC), finding LOD and LOQ of 0.2 and 0.8 μg kg⁻¹, respectively.

Linearity of the Response of the Method. To assess the linearity of the method, an analytical curve was plotted with seven levels of concentration, ranging from 0.33 to 330 μg kg⁻¹ of AITC in cowpea beans. The curve was significant to the linear model \(y = ax + b\) \((P < 0.0001)\). The calibration curve is described as the relation between the theoretical concentration of an analyte in the sample (stimulus) and its chromatographic area (response).35 The method exhibited satisfactory linearity of response at the concentration interval studied, with a coefficient of determination \(R^2\) above 0.99.

Nevertheless, only the coefficient of determination is not enough to assure the linear fitting to the analytical curve. To verify whether the linear fit was adequate to the analytical curve, residuals were plotted and the Cochran test of the data was applied. The calculated Cochran values were lower than the tabulated Cochran value, indicating homogeneous variances as the concentration increased. The error variance was constant in the concentration range studied, with the residues being randomly distributed around zero. This result indicates that, considering the studied concentration range, the model has a homoscedastic behavior.

Accuracy and Precision. The accuracy was evaluated using recovery tests. Blank samples of cowpea beans were spiked.

Figure 5. Univariate optimization of the fortification: flask with the cap (A) or flask without the cap (B), in combination with the extraction time of cowpea samples by the HS-SPME method. Sample mass: 5 g; fiber exposition times: 8, 14, and 20 min by 40 °C; DVB/CAR/PDMS fiber.

Figure 6. In blue, chromatogram obtained from the analysis of beans fortified with AITC \((t_R = 3.0\ \text{min})\) at a concentration of 0.33 μg kg⁻¹ \((a)\) and at a concentration of 3.33 μg kg⁻¹ \((b)\) corresponding to 1 limit of quantification (LOQ) and 10 LOQ. In black, chromatogram with pure ethanol, and in pink, chromatogram of white matrix + ethanol after extraction and analysis by HS-SPME-GC/FID.
Table 1. Comparison of the Proposed Method with Other Analytical Methods for the Determination of Pesticides in Cowpea Beans

| Reference | Compounds | Technique of Sample Preparation | Analysis Technique | Mass of Sample (g) | Extraction Solvent | Cleanup | LOQ $^a$ (μg kg$^{-1}$) | Recovery (%) | CV $^b$ (%) |
|-----------|-----------|---------------------------------|--------------------|-------------------|-------------------|---------|------------------------|--------------|-------------|
| Proposed Method | Allyl isothiocianate | HS-SPME $^c$ | GC/FID $^d$ | 5 | do not need | do not need | 0.33 | 108.2–114.8 | 9 |
| 36 | Multi-residue | ASE $^e$ | GC-MS $^f$ | 5, 36 | acetone/dichloromethane (3:1 v/v), 30 mL of NaCl solution | 20 mL hexane/diethyl ether (94:6 v/v), 30 mL hexane/acetone (v/v) | 1.5–9.0 | 70.0–115.0 | <12.0 |
| 37 | Aldrin, dieldrin, endrin, heptachlor, chlordane, endosulfan | Soxhlet extractor | GC-ECD $^g$ | 20 | dichloromethane | 5 g silica gel, 0.5 cm$^3$ of MgSO$_4$ and 15 mL of n-hexane | 0.85–5.91 | 89.65–98.23 | n.d. |
| 38 | Multi-residue | QuEChERS $^j$ | GC-MS/MS | 10 | 10 mL of ACN $^k$ and 4 g of MgSO$_4$ | 150 mg of MgSO$_4$ | 2.0–8.0 | 74.0–129.0 | <16.4 |
| 39 | Difenoconazole | ME-IL-VALLME $^l$ | HPLC $^{m}$ | 4 | 20 mL of Tween, 30 μL of ionic liquid and 30 μL methanol | do not need | 100.0 | 78.6–94.8 | <9.6 |
| 39 | Difenoconazole | Modified QuEChERS | HPLC-MS/MS | 4 | 20 mL of ACN, 2 g NaCl | 30 mg PSA $^n$ and 20 mg GCB $^{o}$ sorbents | 50.0 | 92.0–118.0 | <3.2 |
| 40 | Multi-residue | SPE $^p$ | GC-ECD | 20 | 40 mL of ACN, 5 g of NaCl | graphitized carbon/SPE, 22 mL of a mixture of ACN and toluene (3:1 v/v) | 10.0 | 76.6–107.0 | <5.0 |

$^a$Limit of quantification. $^b$Coefficient of variation. $^c$Headspace solid-phase microextraction. $^d$Gas chromatography/flame ionization detection. $^e$Accelerated solvent extractor. $^f$Gas chromatography/mass spectrometry. $^g$Sodium chloride. $^h$Gas chromatography/electron capture detector. $^i$Anhydrous magnesium sulphate. $^j$Quick, Easy, Cheap, Rugged, and Safe. $^k$Acetonitrile. $^l$Micellar extraction combined with ionic liquid-based vortex-assisted liquid–liquid microextraction. $^m$High-performance liquid chromatography. $^n$Primary secondary amine. $^o$Graphitized carbon black. $^p$Solid-phase extraction.
with AITC at four concentrations. The relative recovery attained 114.8, 98.2, 108.1, and 109.4% for the concentrations of 1, 10, 50, and 250 times the LOQ value, respectively. For the same levels, the repeatability, given by the coefficient of variation (CV%), was 17.3, 10.5, 5.1, and 3.9%, respectively. The relative recovery for determining pesticide residues is deemed satisfactory within the interval of 70–120%, with repeatability associated with a CV lower than or equal to 20%.32

The intermediate precision (within-laboratory reproducibility) was also assessed through the CV% value, which should score less than 20%.32 In this case, samples of cowpea beans were fortified with AITC at 50 times the LOQ value (16.5 μg kg⁻¹) and then subjected to the method for three consecutive days. Table 1 shows the comparison of analytical performance properties of the proposed method with other methods reported in the literature for the determination of pesticides in cowpea beans. The recovery and CV values of the method are better than or comparable to those reported in some references. The detection limit obtained here was better than those reported (Table 1). Headspace solid-phase micro-extraction (HS-SPME) has advantages of high purity of the extract, not requiring preconcentration and sample cleanup procedures, eliminating the use of organic solvent in the step of extracting the analytes, reduced total sample analysis time, as well as low cost and simplicity per sample.

According to the "Guidance document on analytical quality control and method validation procedures for pesticide residues and analysis in food and feed" (SANTE/11813/2017), the validation must be conducted to provide evidence that the method is suitable for the intended purpose. Thus, the studied methodology is viable for determining AITC in cowpea beans, as it produced results of validation that match the required criteria.32

Persistence of Allyl Isothiocyanate Residue in Cowpea Beans. Periodically, analyses by HS-SPME-GC/FID were performed after the application of AITC in the cowpea bean samples (namely, after 1, 4, 8, 12, 16, 20, 24, 28, 32, 36, 40, 44, and 48 h). Twenty-four hours after fumigation (treatment time), the level of AITC quantified in the grains was 1.6 μL kg⁻¹, which implies that the initial concentration decreased by 93.2%. Thirty-two hours post-treatment, approximately 100% of the AITC waste had already dissipated, which proves its safe use as a fumigant. Moreover, the residual concentration of AITC was not possible to be detected after 36 h, as the trace levels were below the detection limit of the method.

Different mathematical models were adjusted to try to explain the degradation/dissipation of AITC residue in cowpea bean grains (Table 2). The quadratic polynomial model \( y = ax + bx + cx^2 \) presented the best fit, with a coefficient of determination \( R^2 \) equal to 0.97. Figure 7 shows the residual concentration of AITC in cowpea beans 36 h after fumigation.

### Table 2. Mathematical Models Fitted to the Data of AITC Residue in Cowpea Beans Treated by Fumigation

| model          | equation                  | \( R^2 \)  | RMSE \( \mu g L^{-1} \) | \( P \)-value |
|----------------|---------------------------|------------|------------------------|--------------|
| exponential    | \( y = 42.999 e^{-0.16x} \) | 0.9495     | 8.0262                 | <0.0001      |
| linear         | \( y = -0.748x + 20.219 \) | 0.8868     | 2.7683                 | 0.0001       |
| logarithmic    | \( y = -7.291\ln(x) + 25.742 \) | 0.9070     | 2.3349                 | <0.0001      |
| quadratic polynomial | \( y = 0.027x^2 - 1.6286x + 24.626 \) | 0.9737     | 1.2496                 | <0.0001      |
| power          | \( y = 72.122x^{-1.260} \) | 0.6579     | 14228.5                | 1.00         |

\( a \), \( b \), and \( c \) are coefficients obtained from the regression analysis. The determination coefficient \( R^2 \) shows the model's fit to the data. The root-mean-square error (RMSE) is a measure of the magnitude of error in the predictions. The \( P \)-value indicates the statistical significance of the model.

The AITC sorption rate by the grains is a significant factor to be considered when estimating the quantity of product necessary to guarantee effective fumigation.36 The application of pesticides with little to none toxic residue is a crucial factor for the appropriate fumigation in foodstuff. On the other hand, the quick AITC dissipation might compromise its diffusion throughout the whole mass of grains.

Some research shows that botanical insecticides have little persistence in stored grains.41,42 Reactions of oxidation, isomerization, cyclization, and dehydrogenation triggered by enzymes or other molecules, such as oxygen, are common. Temperature, light, and atmospheric oxygen availability also influence the stability of essential oils.43 On that account, the result of the present study is relevant for the development of technologies that could increase the effectiveness of fumigation with AITC for controlling plague insects in stored grains.

### CONCLUSIONS

The method of headspace solid-phase microextraction showed a satisfactory analytical performance regarding the parameters selectivity, linearity, precision, and accuracy. As it does not use an organic solvent, the handling involved in this technique is relatively simple, and the protocol allows making routine analyses of AITC in beans used for consumption.

The evaluation of cowpea bean samples proved that 32 h after the fumigation treatment, 100% of AITC had already been dissipated. Such an outcome evidences the safety of applying this compound as a fumigant in foodstuff.

### MATERIALS AND METHODS

**Reagents and Solutions.** The reagents used in the preliminary tests were toluene (≥99.5% v/v, Vetec, Brazil), acetonitrile (≥99.9% v/v, Sigma-Aldrich, Brazil), ethanol (≥99.5% v/v, Exodo Ciêntífica, Brazil), and octanol (≥99% v/v, Vetec, Brazil). Initially, a stock solution at a concentration of 10 μg mL⁻¹ was prepared by solubilization of the allyl
isothiocyanate (AITC) (>95% v/v, Sigma-Aldrich, Brazil) in ethanol. So, for the phase of optimization of the method and persistence assays, a working solution of AITC was prepared in ethanol at the concentration of 2.28 g L\(^{-1}\). For the method validation step, working solutions at the appropriate concentrations were prepared directly from the stock solution with the same solvent. The solutions were stored in a freezer at a temperature of \(-20^\circ C\) until further use.

**Samples.** All experiments utilized cowpea beans (variety “BRS Guarába”) as a raw material. The beans had 10% moisture content and bulk density of 796 kg m\(^{-3}\). They were cultivated free from insecticides, in the Brazilian state of Maranhão (4° 27’ 18” S latitude, 43° 53’ 09” W longitude, 43 m altitude). After harvest, they were maintained under refrigeration (\(-18^\circ C\)) to prevent any infestation. Ultimately, the grains were packed in nylon bags and taken to the Analytical Chemistry Laboratory (LAQUA), at the Universidade Federal de Viçosa (UFV), where the experiments were conducted.

**Sample Fortification.** Bean samples (5.0 g) were fortified with 50 \(\mu\)L of a solution of allyl isothiocyanate (AITC) at concentrations of 2.28 g L\(^{-1}\), resulting in a final concentration of 22.42 \(\mu\)g kg\(^{-1}\) in the samples. The concentration used corresponds to the lethal concentration (LC) of AITC to kill 95% of the population (LC\(_{95}\)) of *C. maculatus* in cowpea beans determined by bioassays in previous studies.\(^{16}\) The grains were vortex-homogenized (Vortex Mixer CE, Korea) for 1 min, allowed to stand with the jar open for 5 min, and subjected to headspace solid-phase microextraction and analysis by gas chromatography with a flame ionization detector. In the extraction process, we used a 50/30 \(\mu\)m divinylbenzene/carboxen/poly(dimethylsiloxane) (DVB/CAR/PDMS) StableFlex fiber (Supelco, Bellefort). Before defining the extraction conditions of AITC by the HS-SPME-GC/FID technique, some parameters were optimized and best conditions achieved were used to proceed with the studies.

**Chromatographic Conditions.** The identification and quantification of AITC in the cowpea bean samples was carried out in a gas chromatograph equipped with a flame ionization detector (GC/FID) model 2014 (Shimadzu, Japan). The chromatographic separation took place in an HP-5 capillary column (Agilent Technologies), which had the stationary phase composed of 5% phenyl and 95% dimethylsiloxane (30 m x 0.25 mm i.d., 0.10 \(\mu\)m thick film). Nitrogen (99.999% purity, Air Products, Brazil) was used as the carrier gas at a flow rate of 1.8 mL min\(^{-1}\) and a split ratio of 1:5. The temperatures at the injector and the detector were adjusted to 280 and 300 °C, respectively. The initial temperature at the column oven was 45 °C (2 min), and it was set to increase at 20 °C min\(^{-1}\) up to 110 °C (1.8 min), for a 6 min run. The period of thermic desorption was 3.0 min, after which the fiber was removed from the injector. The runs were handled with the CG solution software (Shimadzu, Japan).

**HS-SPME Procedure.** The HS-SPME technique was employed for the simultaneous determination and quantification of AITC in cowpea beans. Samples (5.0 g) were weighed on an analytical balance (Sartorius BP 221 S, Goettingen, Alemanha) in 44 mL amber flasks fitted with a Teflon cap and silicone/PTFE septum. After the fortification with AITC, the samples were homogenized in a vortex mixer for 1 min, and the flasks were allowed to stand for 5 min for solvent evaporation and interaction of the analyte with the matrix. Next, the flasks containing the samples were closed and transferred to a heated water bath with magnetic agitation. The daily conditioned fiber (DVB-CAR-PDMS) according to the manufacturer’s recommendations was exposed in the headspace gas phase for 15 min at the temperature of 30 °C, with the aid of a manual holder proper for SPME (Supelco Bellefort). Following extraction, the fiber was removed from the flask and immediately inserted into a gas chromatography injector, where it remained exposed for 3 min. The analyte was thermally desorbed from the fiber under the carrier gas flow and then led to the chromatographic column.

**Optimization of the Headspace Solid-Phase Micro-extraction Method for Cowpea Bean Samples.** Three commercially available fibers were compared to establish the best conditions for extracting the analyte from cowpea beans via HS-SPME, namely, 100 \(\mu\)m poly(dimethylsiloxane) (PDMS), 65 \(\mu\)m poly(dimethylsiloxane)/divinylbenzene (PDMS/DVB), and 50/30 \(\mu\)m divinylbenzene/carboxen/poly(dimethylsiloxane) (DVB/CAR/PDMS) fibers, from Supelco (Bellefort, PA). The tests were performed in triplicate, and all fibers were preconditioned as instructed by the manufacturer.

Once the fiber with the best chromatographic response was determined, the temperature of the water bath (30, 40, or 50 °C) and the fiber exposure time (3, 9, or 15 min) were optimized using a \(2^3\) scheme with six central points, for a total of 14 assays. These analyses were performed in duplicate (Table 3). Ultimately, the level of the factors was selected based on the preliminary tests.

**Table 3. Central Composite Design Performed to Establish the Best Conditions for Extracting AITC from Cowpea Bean Samples**

| Test | A (temperature °C) | B (time min) |
|------|-------------------|--------------|
| 1    | 30.0              | 3.0          |
| 2    | 50.0              | 3.0          |
| 3    | 30.0              | 15.0         |
| 4    | 50.0              | 15.0         |
| 5    | 40.0              | 9.0          |
| 6    | 40.0              | 9.0          |
| 7    | 40.0              | 9.0          |
| 8    | 30.0              | 3.0          |
| 9    | 50.0              | 3.0          |
| 10   | 30.0              | 15.0         |
| 11   | 50.0              | 15.0         |
| 12   | 40.0              | 9.0          |
| 13   | 40.0              | 9.0          |
| 14   | 40.0              | 9.0          |

\(^a\)Temperature of the water bath with agitation. \(^b\)Time of exposure of the fiber.
To assess the selectivity, the bean samples untreated and treated with AITC were exposed to the extraction method and analysis, and then the resulting chromatograms were compared. The LOD of the proposed method was determined by considering three times the signal of the noise (S/N = 3) baseline obtained for the AITC-free cowpea beans samples (blank) and analyzed by the GC/FID. The LOQ was determined by considering a signal at least 10 times higher than the noise (S/N = 10). The linearity of the response was determined by the evaluation of bean samples fortified at seven different concentration levels (1 × LOQ, 10 × LOQ, 50 × LOQ, 100 × LOQ, 250 × LOQ, 500 × LOQ, and 1000 × LOQ). These assays were executed in triplicate. After the chromatographic injections, an analytical curve (ratio between peak area and compound concentration) was plotted, followed by linear regression.

The precision of the method was investigated via assays of repeatability and intermediate precision. Samples of cowpea beans were fortified at four AITC levels (1 × LOQ, 10 × LOQ, 50 × LOQ, and 250 × LOQ) in seven replicates, and the repeatability was determined by the coefficient of variation (CV%) of the concentrations obtained experimentally by HS-SPME-GC/FID.

To determine the intermediate precision (within-laboratory reproducibility), cowpea beans samples were fortified with AITC at the level of 50 × LOQ, on three different days. On each of those days, seven identical samples were analyzed by the same analyst, in the same laboratory, and under identical working conditions. The result was considered by the coefficient of variation (CV%). The identification of the compound was performed by comparing the retention time (tR) of the peak with the retention time of the standard solution of AITC in ethanol. Matrix-matched calibration was commonly used to compensate for matrix effects. The accuracy of the method was assessed through the assays of relative recovery, in which a percentage was calculated by the ratio between the average concentration found in the repeatability and the concentration corresponding to the theoretical value multiplied by 100.

**Application of the Headspace Solid-Phase Micro-extraction Method for Evaluating the Persistence of Allyl Isothiocyanate in Cowpea Beans.** The optimized and validated method was employed to assess the persistence of AITC residues in cowpea bean samples. For the experimental part, bean samples (240 g) were put into 0.3 L glass flasks, where they were fumigated with AITC at 22.42 μL kg⁻¹, the lethal AITC concentration to kill 95% of the C. maculatus population (LC95), as it had been determined in previous bioassays.

At the upper part of the flask, a 1.0 cm diameter PVC pipe covered with organza was placed to simulate a plenum. A piece of filter paper moistened with AITC was put on top of this adapted structure, so as to fortify the samples. After that, the flasks were closed with a metallic screw cap and sealed with Parafilm (PM996). They were stowed in an incubator chamber (Lucadema, São Paulo, Brazil) set at a constant temperature of 27°C.

The fumigation testing lasted 24 h overall, but the AITC residue was quantified up from the first hour via the validated HS-SPME-GC/FID method. In each evaluation time (1, 4, 8, 12, 16, 20, and 24 h after the beginning of fumigation), the flasks containing cowpea beans were homogenized, and the samples were quickly weighed and then subjected to extraction and gas chromatography analysis. Twenty-four hours after the fumigation, the grains were transferred to nylon bags (12 × 16 cm) to simulate the usual storage conditions, and once again placed inside the incubator chamber. The residue analysis was repeated every 4 h, until no trace of AITC could be detected in the grains. These experimental procedures were executed in triplicate, and, at every interval, two 5.0 g samples were taken from each flask and separately submitted to extraction and analysis.

AITC persistence was calculated through the analytical curve of the method. In this case, different models were considered to explain the dissipation of the AITC residues in the cowpea beans.

**Statistical Analysis.** The chromatographic responses of the SPME fibers exposed to AITC, considering the different solvents and rest time conditions, were subjected to analysis of variance (ANOVA) and the means were compared by Tukey’s test (P > 0.05). Both time and temperature during extraction (2² factorial scheme) were submitted to ANOVA and regression analysis of the response surface. The calibration curve for AITC extraction and the curves for AITC residue persistence in cowpea beans were also evaluated through ANOVA and regression analysis. The analyses were handled by the software Statistical version 10 (StatSoft, Inc, Tulsa, OK), and the graphs were plotted with the software SigmaPlot 13.1 (Systat Software, Inc., San Jose, CA).

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