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Quantitative analysis of *S*-Allylcysteine in black garlic via Ultra-High-Performance Liquid Chromatography-Tandem Mass Spectrometry

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ABSTRACT
The biological effects of black garlic are stronger than those of fresh garlic, but despite the increasing manufacture of black garlic products, no specifications for controlling their quality have been identified. The most commonly used biological marker of quality is *S*-allylcysteine (SAC), which is a core active compound in the aforementioned products. SAC content is typically determined using high-performance liquid chromatography with ultraviolet detection, but this method presents many problems because of its complex matrices. To determine the content of SAC in black garlic, this study established a sensitive and reproducible method grounded in ultra-high-performance liquid chromatography coupled with tandem mass spectrometry. The instrument used is equipped with a triple quadrupole mass detector with an electrospray ionization source. The analysis involved solid-phase extraction using a strongly acidic cationic exchange agent, which enabled the direct examination of samples without the need for derivatization and the determination of SAC content in 5 min.
The established method was fully validated in terms of linearity, sensitivity, precision, repeatability, and recovery and was successfully applied in characterizing the quality of some black garlic products manufactured in Vietnam. The proposed procedure exhibited high linearity (R^2 = 0.9993) at a limit of detection of 0.25 ng/mL, which is lower than those of other assay techniques. The results indicated that various black garlic products have different SAC contents. This study may be useful in the development of standards for the quality of black garlic.

Keywords: *S*-allylcysteine, Black garlic, UPLC-MS/MS.

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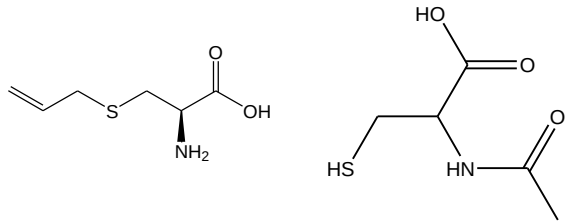
INTRODUCTION
Black garlic is made by storing fresh garlic at a certain temperature and humidity for several days. Its physical properties vary extensively from those of fresh garlic. One such difference is the concentration of *S*-allylcysteine (SAC), which is one of the main biological compounds in black garlic [10,19] and the fresh variant. The latter has only small amounts of SAC, reaching about 20 to 30 µg/g, whereas black garlic has five to six times higher this concentration. SAC is formed via the hydrolysis of γ-glutamyl-*S*-allyl cysteine using the enzyme γ-glutamyl transpeptidase [19]. A significant increase in the SAC content of black garlic is an important change that occurs during its production. SAC is high in antioxidants and can suppress the formation of free radicals, such as the hydroxyl radical and the peroxyl radical [20,21]. Tests on diabetic mice suggested that SAC reduces oxidative stress [22] and blood glucose levels [23]. It also inhibits the development of ovarian cancer, induces ovarian cancer cell death in vitro [24], and suppresses the development of prostate cancer in in vivo trials [25]. SAC is used to treat nasopharyngeal cancer [26] and may protect neurons against cell death [27]. It can likewise serve as an anti-inflammatory agent, protecting the kidneys from diabetes mellitus [28]. Because it occurs in relatively high amounts and because of its stability and many therapeutic effects, SAC can be employed as a biological marker of the quality of black garlic [19]. SAC can be quantified in fresh garlic via high-performance liquid chromatography with ultraviolet detection (HPLC-UV), but such method is ineffective for determining SAC content in black garlic because its chemical composition is more complex than that of the fresh variety, and many UV-absorbing products render baseline levels unstable, thereby yielding inaccurate results [29]. Instead, SAC in black garlic can be quantified at high reliability through HPLC with fluorescence detection (FLD) [19,29,30], HPLC-mass spectrometry (MS) without the use of derivatives [17], HPLC with post-column derivatization [31], and LC-tandem mass spectrometry (MS/MS) with post-column derivatization [32]. Among these approaches, the most reliable are HPLC-FLD, HPLC-MS, and LC-MS/MS. Fluorescence detectors are uncommon in many laboratories, and their use requires carrying out derivatization with a fluorescence agent. Mass spectrometers are increasingly adopted because of their sensitivity and selectivity, and MS/MS using a triple quadrupole mass detector enables analysis without going through a derivatization step. These features prompted the quantification of SAC in black garlic through LC-MS/MS with *N*-acetylcysteine (NAC) as the internal standard, and the mobile phase was varied in the isocratic mode. Carrying out solid-phase extraction (SPE) with a strongly acidic cationic exchanger in sample preparation enables direct analysis without derivatization and analysis completion in 5 min.

EXPERIMENTAL

2.1. Chemicals, reagents, and materials

SAC (over 98%) was purchased from Sigma-Aldrich (Steinheim, Germany), and NAC (over 99%) was provided by the Institute of Drug Quality Control of Ho Chi Minh City (Vietnam). The structures of these compounds are presented in **Figure 1**. HPLC-grade methanol was derived from JT Baker (United States), and HPLC-grade ultrapure water was obtained from Merck (Darmstadt, Germany). The ammonium hydroxide used was of analytical grade (Xilong, China). Eight black garlic products, which are all of the one-clove variety, were collected from a market in Can Tho City, Vietnam. Strata SPE strongly acidic cationic exchange cartridges (500 mg, 3 mL) were acquired from Phenomenex (Torrance, USA).



S-allylcysteine *N-acetylcysteine*

Figure 1. Chemical structures of *S*-allylcysteine and N-acetylcysteine

2.2. Preparation of standard solutions

A certain amount of SAC and NAC were dissolved in water to prepare two stock solutions, and standard solutions were prepared by diluting the stock solutions with water to mixtures with a series of appropriate concentrations falling in the range of 1 to 100 ng/mL. The standard solutions were then filtered through a 0.22 µm membrane prior to injection.

2.3. Sample treatment

The black garlic was crushed and homogenized. An aliquot (0.2 g) of a sample was sonicated with 10 mL of water for 30 min at room temperature using an ultrasonic water bath (50 kHz). This procedure was repeated three times. The first and second extractions were collected (A), whereas the third extraction was separated (B). All the extractions were filtered and reduced in volume by heating at 70°C in a water bath to approximately 4 mL before injection into the SPE tubes. Samples A and B were loaded separately onto the Stata SPE cartridges, which were preconditioned sequentially with 6 mL of methanol and 6 mL of ultrapure water. The cartridges were then washed, again with 16 mL each of the ultrapure water and methanol. Analytes were eluted by passing 4 mL of 7% ammonium hydroxide in methanol, after which the eluent was evaporated to dryness at 70°C in a water bath. The residue was dissolved in water to yield a 100 mL mixture, which was subsequently diluted 10 times with water then filtered through a 0.22 µm membrane. The final solution of sample A was incorporated with NAC to a concentration of 50 ng/mL and was injected into the UPLC system for analysis. The final solution of sample B was used as a blank sample. Each sample was analyzed at least three times at an injection volume of 10 µL.

2.4. UPLC-MS/MS conditions

Chromatographic analyses were performed on a Waters Acquity UPLC H-class system (Waters Corp., Milford, MA, USA) consisting of a binary pump solvent management system, an online degasser, and an autosampler. A Synergi Hydro-RP C18 column (250 × 4.6 mm; 4 µm) was used in all the analyses. The mobile phase was tested with methanol-water and acetonitrile-water at a flow rate of 0.8 mL/min and a column temperature of 25°C. Mass spectrometry was carried out using an Xevo TQD triple quadrupole mass detector (Waters Corp., USA) equipped with an electrospray ionization (ESI) source. ESI-MS spectra were acquired in positive ion multiple reaction monitoring (MRM) mode. The conditions of MS analysis were as follows: a capillary voltage of 3.5 kV and a desolvation gas flow rate set to 1000 L/h at a temperature of 500°C. The cone voltage and collision energy were set to match the MRM of each analyte, and the dwell time was 0.025 s. The MS/MS detection parameters are summarized in **Table 1**.

Table 1. Retention times and related MS data on SAC and NAC detected via UPLC-MS/MS

Analyte	Retention times (min)	Precursor ion (m/z) $[M+H]^+$	Product ion (m/z)	Cone voltage (V)	Collision energy (V)	Purpose
SAC	3.73	162.08	145.00	16	8	Quantification
			72.91	16	12	Identification
NAC	2.48	164.19	122.01	20	4	Quantification
			75.97	20	16	Identification

2.5. Stability and matrix effects

Standard NAC and SAC solutions in water and in the blank sample were stored at 25°C and injected into the apparatus at 0, 2, 4, 8, 12, and 24 h to evaluate the stability of the solutions. The regression curves of the water and blank sample were established on the basis of seven concentrations. The difference in the slope of two equations was calculated to determine matrix effects.

2.6. Method validation

2.6.1. Selectivity

The standard solutions, blank sample, blank samples spiked once to twice with the standard solutions were examined to identify interference peaks at the retention time of SAC.

2.6.2. Calibration curves, limits of detection (LOD), and limits of quantification (LOQ)

The peaks were plotted against corresponding concentrations to obtain calibration curves. LOD and LOQ values were determined using diluted standard solutions at analyte signal-to-noise (S/N) ratios of about 3 and 10, respectively. The S/N ratios were calculated by dividing peak height by the background noise value.

2.6.3. Precision and accuracy

Intra- and inter-day variations were investigated by determining analytes in six replicates within one day and duplicating the experiments on three consecutive days. Variations in peak areas were taken as measures of precision and expressed as percent relative standard deviations (%RSDs). This method's accuracy was evaluated using a recovery test, which was performed by adding known amounts of the standard solutions to 10, 20, 30, and 40 ng/mL of black garlic samples. Each level was operated with four samples. The spiked samples were then extracted, processed, and quantified in accordance with the aforementioned method. The average percentage of recovery was calculated using the following formula: recovery (%) = (total observed amount − original amount) × 100% / spiked amount.

2.7. Identification and quantification

Target peaks were identified through a comparison of their retention times and mass/charge ratios (m/z) with those of the standards. Quantification was performed using linear calibration plots of peak area ratios and concentrations.

RESULTS AND DISCUSSION

3.1. Sample treatment

Water was chosen as a medium for extracting SAC from the black garlic because this substance is soluble in water but insoluble in organic solvent. This decision is in line with studies on the quantification of SAC in garlic or black garlic [19,29,30]. Given that SAC is a weak basic amino acid, which differs from other impurity groups, impurities in the compound can be removed using a cationic exchange column. Two types of cationic exchange SPE agents are available in the market: strongly acidic cationic exchangers with benzene sulfonic acid and weakly acidic cationic exchangers with carboxylic acid. A strongly acidic cationic exchange column is suitable for weak bases, whereas weak cationic exchange columns are appropriate for base substances, usually strong ones. Complexed extracts should be investigated using both types of columns.

SAC was assumed to have been retained on the column used in this work, and impurities were washed off. Then, an alkaline agent was used to remove the SAC from the column. The washing solvents were water and methanol, and the solvent for SAC elution was 7% NH_4OH in methanol. The results showed that the strongly acidic cationic exchange SPE column could be satisfactorily eliminated from the mixture. The eluent was washed with 4 mL of 7% NH_4OH in methanol, which is a volume sufficient to remove all the SAC from the column. The result was perfectly matched, as found in the rechecking based on the mass spectra. The third extract did not contain SAC but still had other residues. Thus, after treatment with the SPE column, the extract could be used as a blank sample for analysis.

3.2. Optimization of chromatographic conditions

Preliminary experiments involving several mobile phase solvents, such as ACN, MeOH, H_2O, and a mixture thereof, were conducted to obtain high efficiency and separation. A 40:60 (v/v) mixture of methanol and water was found to be the best (**Figure 2 and Figure 3**) and could elucidate impurities better than a 20:80 (v/v) mixture of the aforementioned substances. The latter allows subsequent injections to be unaffected by remaining impurities. Mass spectrometry is a highly efficient method and therefore enables the recognition of analytes and impurities on the basis of mass, but if a previous injection is not washed off a column after several injections, the ion capture capacity of the probe may diminish because of excessive ionic competition, which leads to mistaken identification. Moreover, a dirty column affects the retention time of a substance. Accordingly, the SAC peak was split under a mixture of acetonitrile and water (5:95, v/v). This result may be due to the fact that the transfer of SAC between the mobile and stationary phases was incomplete. A small fraction of SAC was separated first, thereby forming a small peak early in the chromatogram. Owing to the insolubility of SAC in acetonitrile, the amount of acetonitrile in the mobile phase affected the SAC distribution. In addition, this solvent mixture may not be suitable for SAC analysis via mass spectrometry, resulting in incomplete ionization and non-replicative analysis.

Previous studies used a gradient elution program with a mobile phase containing pH-adjusting agents in analyzing SAC. In our SAC examination, a simple mixture of methanol and water was used, which ensured the accuracy and correctness of the analysis and considerably simplified such exploration.

3.3. Stability and matrix effects

The results of matrix effect tests showed a significant influence on the analytes (45.35%), signifying the need to construct a standard calibration curve in a blank sample to reduce matrix effects. Although the sample matrix was very complex, the mass spectrometer still analyzed the SAC, which is advantageous for the high specificity of mass spectrometry. The stability analysis of the standard solutions showed that their content would decrease considerably after 24 h. The NAC was stable for up to 8 h, whereas the SAC was in this condition for up to 4 and 2 h when diluted in water and the blank sample, respectively. Given that the blank sample may have also contained an alkaline substance after treatment with SPE or many enzymes, as well as organic acids from the extract, the SAC in the blank sample was less stable in water. Ensuring accurate analytical results therefore necessitates the analysis of solutions within 2 h after preparation.

3.4. Method validation

The established UPLC-MS/MS method for the quantitative determination of SAC was validated by determining its selectivity, linearity, LOD, LOQ, and intra-day and inter-day precision and accuracy. The blank sample did not have a SAC

signal, whereas the blank sample spiked with the standard solutions once exhibited a SAC signal at a retention time equivalent to one such period for SAC in the standard solutions. The blank sample spiked twice with the standards showed a peak SAC area that is significantly higher than that of the blank spiked once with the standards. The mass spectra of MS1 and MS2 were discernible from the complex matrix, as was the case with the black garlic, with the method still yielding the mother ion and the two daughter ions of the analytes, thus satisfying the required number of ion points. The calibration curve (y = 0.22213x + 0.068283) showed good linearity (r = 0.9996) within the test range of 1 to 100 ng/mL, and the LOD and LOQ were 0.05 and 0.25 ng/mL, respectively. The %RSD values of intra- and inter-day variations were all less than 1.51% (**Table 2**), and the overall recovery fell between 97.08% and 102.96% with %RSDs of less than 2.53% (**Table 3**). All the results pointed to the accuracy of the approach put forward in this work

3.5. Application

All the black garlic samples contained SAC (**Table 4**), but only one had a SAC content of 100 µg/g. Three samples had a SAC content greater than 250 µg/g, and four showed a SAC content exceeding 300 µg/g. Previous studies found SAC contents ranging from 85 to 200 µg/g [17,19,33], showing that the black garlic in Vietnam have the same or higher SAC levels. This is a good sign of the quality of black garlic produced in Vietnam.

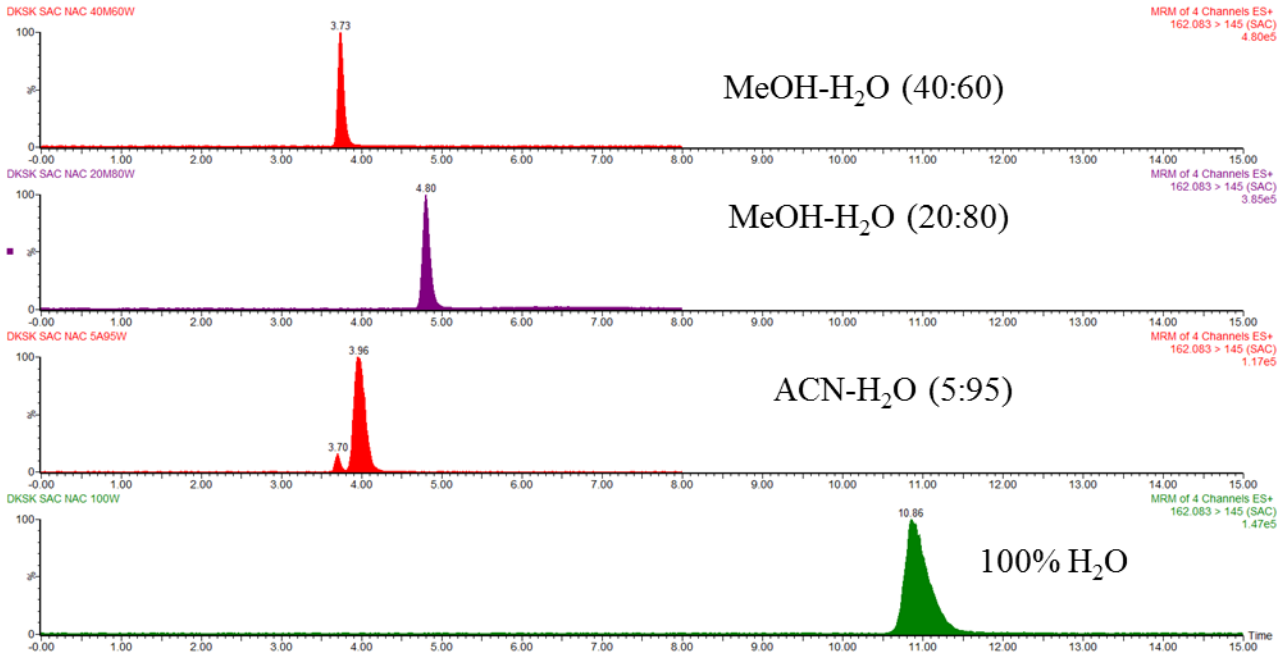


Figure 2. Chromatograms of SAC at different mobile phase conditions

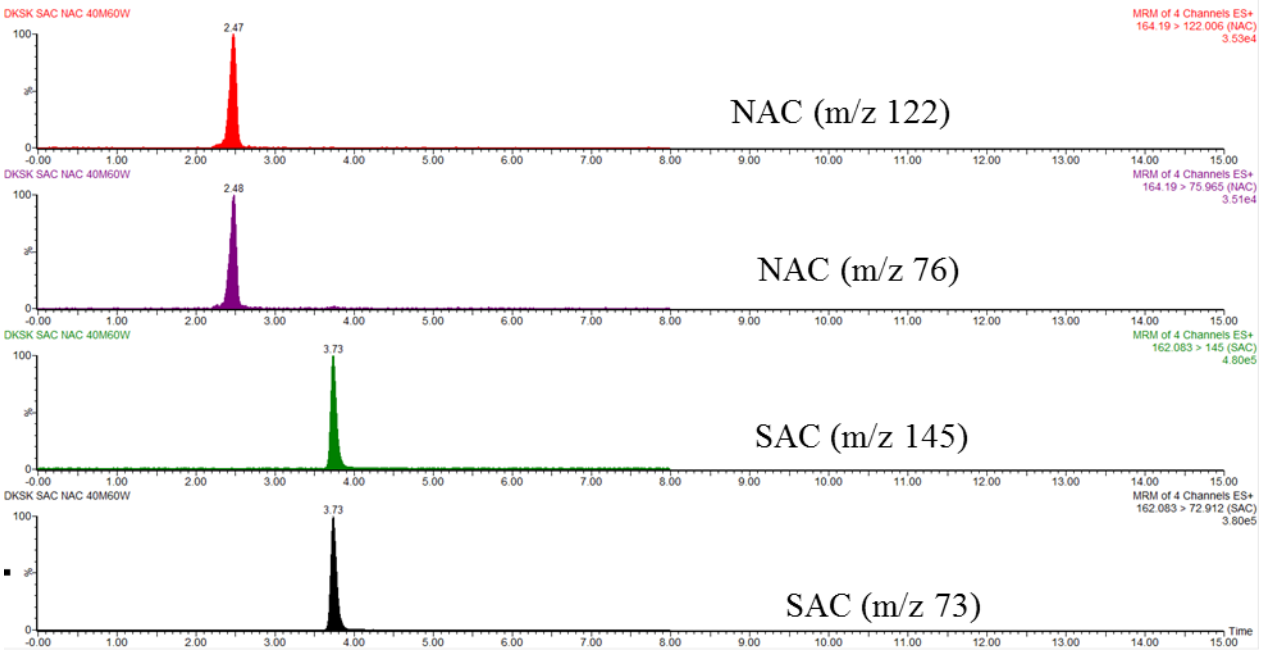


Figure 3. Chromatograms of SAC and NAC at MeOH-H2O (40:60, v/v)

Table 2. Precision results

n = 6	Day 1	Day 2	Day 3
Average content of SAC in black garlic (µg/g)	100.24	100.43	102.39
RSD	1.45%	1.51%	0.56%

Table 3. Recovery results

n	10 ng/mL	20 ng/mL	30 ng/mL	40 ng/mL
1	100.00%	102.75%	101.15%	102.96%
2	98.33%	98.12%	97.95%	101.47%
3	100.69%	98.05%	100.77%	97.08%
4	99.14%	100.88%	98.22%	101.56%
RSD	**1.03%**	**2.29%**	**1.67%**	**2.53%**

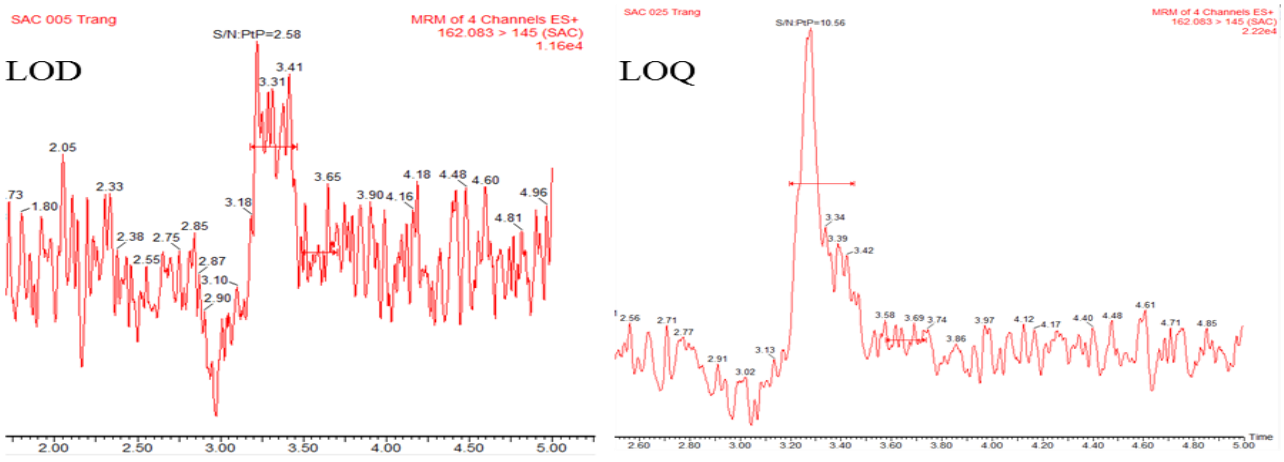


Figure 4. LOD and LOQ of SAC at 0.05 and 0.25 ng/mL, respectively

Table 4. Contents (µg/g) of SAC in several black garlic samples (n = 3)

No. of samples	SAC ± SD (µg/g)	No. of samples	SAC ± SD (µg/g)	No. of samples	SAC ± SD (µg/g)	No. of samples	SAC ± SD (µg/g)
1	346.09 ± 4.93	3	95.07 ± 1.84	5	272.86 ± 4.84	7	291.98 ± 30.35
2	427.05 ± 3.56	4	308.00 ± 22.17	6	312.13 ± 3.42	8	348.03 ± 92.90

CONCLUSION

In this research, a UPLC-MS/MS method was established and validated as a reliable and accurate approach for the qualification and quantification of SAC in black garlic within 5 min. The data showed remarkable differences in SAC content among the black garlic samples but overall reflected a very high SAC in the black garlic coming from Vietnam. The results reflected this work's potential contribution to quality control for the production of black garlic.

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