



## Investigation of Allicin Stability in Aqueous Garlic Extract by High Performance Liquid Chromatography Method

Kenneth Chong<sup>1</sup>, Martha P. Zamora<sup>2</sup>, Dileshni A. Tilakawardane<sup>2</sup>,  
Nancy E. Buckley<sup>2</sup>, James A. Rego<sup>1</sup> and Yan Liu<sup>1,2</sup>

<sup>1</sup>Department of Chemistry and Biochemistry, California State Polytechnic University Pomona, 3801 West Temple Avenue, Pomona, CA 91768, USA.

<sup>2</sup>Department of Biological Sciences, California State Polytechnic University Pomona, 3801 West Temple Avenue, Pomona, CA 91768, USA.

### Authors' contributions

*This work was carried out in collaboration between all authors. Authors KC and YL carried out the HPLC analysis of allicin. Authors MPZ, DAT and NEB sampled garlic cloves and made the dilutions of garlic extract samples. Author JAR synthesized and characterized allicin. In addition, author YL prepared the manuscript. All authors read and approved the final draft of the manuscript.*

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### ABSTRACT

**Aims:** The aims of this work were to quantify allicin - an abundant antioxidant in different aqueous garlic extract samples and to determine the stability of allicin in the extract under different thermal and solvation conditions.

**Study Design:** Descriptive research.

**Place and Duration of Study:** Department of Chemistry and Biochemistry and Department of Biological Sciences, California State Polytechnic University, Pomona, between May 2013 and September 2014.

**Methodology:** A reversed-phase high performance liquid chromatography method was used to analyze allicin in garlic extracts. Quantification of allicin in garlic extracts was made by comparing its peak area with the standard calibration curve of allicin with known concentrations. The kinetic study of allicin degradation in garlic extracts was based on the Arrhenius theory.

**Results:** The amount of allicin in aqueous garlic extracts varied from 35.6 to 44.5 mM. The thermal stability study shows that the degradation of allicin is a first-order reaction and the preliminary data shows that the degradation of allicin in different solvents has different kinetic parameters.

**Conclusion:** The concentration of allicin in garlic extract samples was similar to each other, despite of different sampling dates. For medicinal purpose, garlic extracts should be used freshly to prevent any degradation of allicin.

*Keywords: Allicin; high performance liquid chromatography; garlic extract; quantification; thermal stability; chemical stability.*

## 1. INTRODUCTION

Garlic, a common food that is well known for its characteristic flavor, has long been used for medicinal purpose to fight against cancer and cardiovascular disorders. [1,2] Garlic has the potential to decrease platelet aggregation, lower total plasma cholesterol, reduce blood pressure in clinical trials, and modulate the immune system. [1-5] In addition, garlic is known to have antimicrobial activity. [6] Many investigators have revealed that the pharmacologic and therapeutic effects of garlic come from allicin, a diallyl thiosulfinate compound found in the fresh garlic extract. [7,8] Although allicin represents about 70% of the overall thiosulfates present or formed upon crushing the garlic cloves, [9] allicin is not actually present in whole garlic bulbs. When garlic cloves are crushed, cut, or chewed, the pressure ruptures the cell membranes. S(+)-allyl-L-cysteine sulfoxide (alliin) inside the cells quickly degrades into allicin and other sulfur-containing compounds with the catalytic action from the enzyme allinase. [10,11] Pharmacologically, allicin is active against many bacteria and viruses. [12,13] In addition, allicin prevents lipid peroxidation and has an outstanding antioxidant activity. [14,15] The extremely easy access for human beings to garlic cloves and garlic products makes allicin a great contestant for a common antibiotic agent in our daily life.

To take advantage of all these valuable merits of garlic-derived allicin, modern technology is required to identify, quantify, and study the stability of allicin in garlic extracts or commercial dietary supplemental garlic products. Many components of garlic powders, garlic flakes, and aged garlic extracts, including allicin and its precursors, have been determined by high performance chromatography (HPLC), gas

chromatography (GC), HPLC coupled with mass spectrometry (HPLC-MS), and GC-MS. [16-18] Nine organosulfur marker compounds were detected and identified in an aged garlic extract with solvent extraction and GC-MS analysis. [16] Diallyl sulfides in garlic powder was analyzed by LC-MS with Ag(I) induced ionization. [19] As for the identification of allicin, GC has been applied to the indirect quantification of allicin by converting it to either diallyl disulfide or allylmercaptan. However, GC methods require allicin as an external standard. Han et al reported a spectrophotometric method for quantitative determination of allicin and total garlic thiosulfates based on the reaction of allicin with S-allylmercaptocysteine and requiring no allicin standard to quantify. [11] Since then, HPLC has been predominantly employed in the allicin quantification. [20,21] In addition, HPLC is the most versatile and widely used elution chromatography and it gives high-resolution separations with a wide variety of techniques. [22] The quantitative determination of allicin in garlic was made possible by the supercritical fluid extraction with CO<sub>2</sub> and the standard addition of alliin, and HPLC with UV-vis absorbance detection. [23] Mochizuki et al reported a rapid method base on UV with HPLC for quantitative determination of allicin in aqueous garlic extract. [24] A simple, rapid, and reliable HPLC method is still desired in the quantification of allicin in garlic samples.

Although a few reports have described allicin as being relatively stable in an aqueous and ethanolic solution, the decomposition of allicin is an undeniable fact. [25,26] Some commercially available allicin standards do not contain the purported concentration due to decomposition. [23] Allicin reacts very rapidly with free thiol groups present in garlic matrices. The thermal conditions and the chemical environment where

allicin is present greatly affect the stability of allicin. It was reported that the antibacterial use of garlic was influenced by the chemical stability of thiosulfinates. [27] Some work has been done to determine the thermostability of allicin in an ordinary garlic extract by systematic analyses employing chemical and biological assays. The kinetic parameters for the decomposition processes of allicin in the selected garlic extracts obtained with ethanol and acetone have been determined, and different reaction orders have been reported in the different solvent. [26] As for other solvents, the kinetic picture is not clearly defined. Thus, the objectives of this work were to identify and quantify allicin in different garlic samples by HPLC method first, and then determine the thermal and chemical stability of allicin in fresh garlic aqueous extract in different solvents. This information is necessary to determine the activity, formulation, and dosage of a natural remedy taken from garlic extracts.

## 2. EXPERIMENTAL DETAILS

### 2.1 Chemicals and Apparatus

Alliin was purchased from Sigma-Aldrich (St. Louis, MO). Methanol, acetone, and dimethyl sulfoxide (DMSO) of HPLC grade were purchased from Fisher Scientific (Pittsburg, PA). Pyrogen-free water was purchased from Hospira (Lake Forest, IL). All chemicals are used as received without further purification. Allicin was synthesized by the peroxyacid oxidation of diallyl disulfide according to the procedure of Small et al. [28] Due to the thermal instability of allicin, the crude product was purified by silica gel chromatography rather than distillation.

### 2.2 Garlic Extract Sample Preparation

The aqueous garlic extract was prepared as previously described. [29] Briefly, garlic purchased from the local grocery store was combined with pyrogen free water (PFW) (0.5g garlic/ml PFW) and ground using a Waring blender for one minute. The homogenate was passed through grade-50 cheesecloth and subsequently filter-sterilized using a Nalgene 0.2 $\mu$ m pore-sized filter unit (NalgeNunc International, Rochester, NY) connected to a vacuum pump. The flow-through from the Nalgene filter was aliquoted into microcentrifuge tubes and stored at -80°C until used. Garlic extract sample stocks and the standard allicin were diluted with pyrogen-free water as needed, prior to HPLC analysis.

### 2.3 Quantification and Stability Study of Allicin in Garlic Extract Samples

Allicin in the garlic extracts was quantified by a HPLC method which involved using a Zorbax Eclipse XDB-C18 column with a size of 4.6 mm x 150 mm, A G1312A bin pump, and a G1315A UV detector. Above parts were included in the Agilent-1100 HPLC system (Agilent Technologies, Santa Clara, California, USA). Allicin or allicin-containing garlic extracts were applied onto the column and eluted by the isocratic solvent of water/methanol (50/50) at a flow rate of 0.75 mL/min. The absorbance of allicin or garlic extract was monitored at 254 nm. Quantification of allicin in garlic extract was made by comparing its peak area with the calibration curve of the standard allicin with known concentrations.

Different thermal and chemical conditions were employed to investigate the stability of allicin in the garlic extract. Three different temperatures, namely 4, 37, and 100°C, were used to determine the thermal stability of allicin in the garlic extracts. In addition, the stock solution of garlic aqueous extract was diluted in different solvents, including water, methanol, acetone, and DMSO, in order to investigate the chemical stability of allicin in garlic extract.

## 3. RESULTS AND DISCUSSION

### 3.1 Construction of Calibration Curve

To construct the calibration curve, ten standard allicin solutions with different concentrations were used with each solution being analyzed repeatedly six times. The detector responses of allicin were plotted as function of the allicin concentration to construct a calibration curve for allicin quantification. A linear relationship was observed between the detector responses and the allicin standard concentrations ranging from  $1.72 \times 10^{-4}$  M to  $5.68 \times 10^{-2}$  M. A least squares regression analysis equation was determined to be:

$$\text{Allicin signal} = 24376 \times \text{allicin concentration} + 8.4$$

The relative standard deviation of the measurements ( $n = 6$  for each concentration) was less than 3.0%. The  $R^2$  value of 0.9987 was found for the linear fit equation. When the concentration of allicin dropped below  $1.72 \times 10^{-4}$  M, no significant difference between allicin

standard signal and baseline noise was observed. The limit of detection/quantification of our method ( $1.72 \times 10^{-4}$  M) is sufficient for the analysis of the majority of garlic products.

### 3.2 Quantification of Allicin in Garlic Extract Samples

A same brand of garlic cloves (not necessarily the same batch) purchased from a local grocery store at different times (from spring to winter) was used to prepare garlic extract samples. All garlic extract samples were made at different times, as well. A representative chromatogram of a garlic aqueous extract sample analyzed by the developed HPLC method is shown as Fig. 1. A few peaks were observed on the chromatogram of a garlic extract sample indicating the presence of different compounds. The peak identity of allicin (the third peak with a migration time of 1.23 min) was confirmed by comparing the migration time of the allicin standard solution with that of the garlic extract components in the sample. When a small amount of allicin standard solution was added to the garlic extract sample, the peak area increase was only observed on the third peak on the chromatogram further confirming the peak identity of allicin. The quantification results for different garlic extracts based on the standard calibration curve are listed in Table 1. All garlic extract samples contained similar amount of allicin varying from 35.6 to 44.5 mM despite of different garlic cloves and different preparation times.

**Table 1. Quantification of allicin in garlic extracts**

Sample number	Allicin conc. (mM)
S1	36.8
S4	39.5
S5	35.6
S18	44.5
S43	36.6

*\*sample number was randomly selected and was not specific to any sampling dates*

Although the HPLC separation condition for all compounds is not optimal and ideal, the allicin peak is well resolved from the peak cluster with a resolution of 1.54 from its neighboring peak. Lowering the flow rate of the mobile phase effectively resolved the components in the first few peaks. Up to ten peaks were observed when the flow rate was decreased to 0.50 mL/min but

the overall analysis time was much longer. In this project, allicin in the garlic extract is the analyte of interest. Therefore, no further investigation was carried out to identify and increase the resolution of other components except allicin. Compared to other methods such as the Pico Tag derivatizing method, [21] this method can not quantify very low concentration of allicin. However, it requires no particular sample preparation procedures and the filtered garlic extract can be directly loaded onto the chromatographic column for analysis. More importantly, the analysis time is shortened to within 5 min compared to previously reported 12 min. [10,30] The shortened analysis times are essential for real-time monitoring of allicin at the elevated temperature where the natural degradation of allicin occurs rapidly.

### 3.3 Kinetic Study of Allicin Degradation

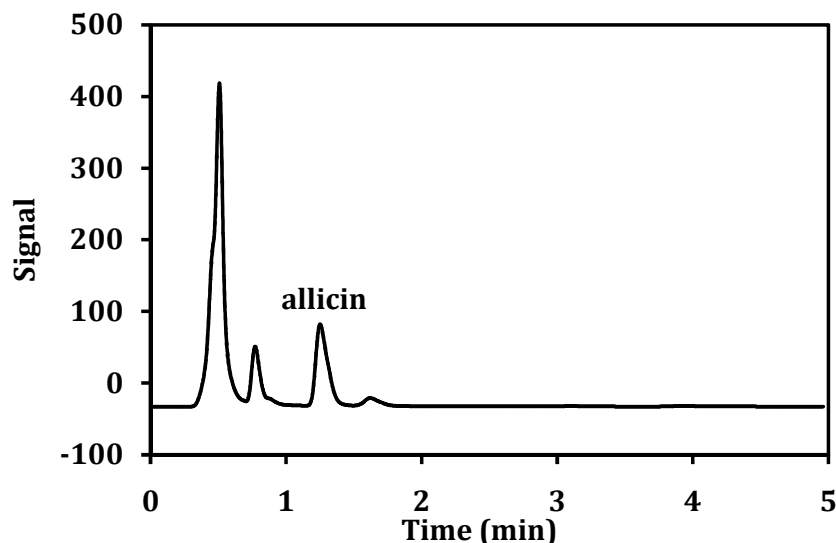
It has been documented that allicin naturally decomposes under normal experimental condition. [31,32] To investigate the kinetics of the degradation process of allicin in the aqueous garlic extract, the concentration of allicin was monitored over a long time period at a constant temperature. To increase the reaction rate of the allicin degradation, the garlic extract was incubated at 100 °C. As demonstrated in Fig. 2a, the degradation of allicin started immediately at the boiling temperature and the concentration of allicin dramatically decreased over the experiment period. By 50 min, the amount of allicin dropped below the detectable threshold of our method. The reaction rate of the allicin degradation can be expressed as the change in its concentration per unit time. Mathematically,

$$-\frac{d[\text{Allicin}]}{dt} = k[\text{Allicin}]^n \quad (1)$$

Where  $k$  is the rate constant,  $n$  is the reaction order,  $[\text{Allicin}]$  is the allicin concentration in the garlic extract and  $t$  is the reaction time. The reaction order,  $n$ , can be mathematically determined by integrating Eq. 1. There are different relationships among the monitored concentrations upon the differences in reaction order [33].

If  $n = 1$ ,

$$\ln[\text{Allicin}] = \ln[\text{Allicin}]_0 - kt \quad (2)$$



**Fig. 1. Representative chromatogram of a garlic extract sample with the third peak being identified as allicin. HPLC conditions: Zorbax Eclipse XDB-C18 column with a size of 4.6 mm x 150 mm, 10  $\mu$ L injection, mobile phase, water/methanol (50/50); flow rate, 0.75 mL/min; UV absorbance wavelength, 254 nm**

If  $n = 1.5$ ,

$$\frac{1}{\sqrt{[Allicin]}} = \frac{1}{\sqrt{[Allicin]_0}} + kt \quad (3)$$

If  $n = 2$ ,

$$\frac{1}{[Allicin]} = \frac{1}{[Allicin]_0} + kt \quad (4)$$

$[Allicin]_0$  in all three equations mentioned above stands for the initial concentration of allicin in the garlic extract when  $t = 0$ . As determined in the previous section, the signal of allicin is linearly proportional to the concentration of allicin. Therefore, the Eq. 2 can be rewritten as:

$$\ln(Allicin\ Sig.) = \ln(Allicin\ Sig.)_0 + k't \quad (5)$$

When the data in Fig. 2a were analyzed, a linear response was obtained in  $\ln(Allicin\ Signal)$  vs. time (demonstrated in Fig. 2b), which indicates the allicin degradation process in the garlic extract at 100°C is a first order reaction.

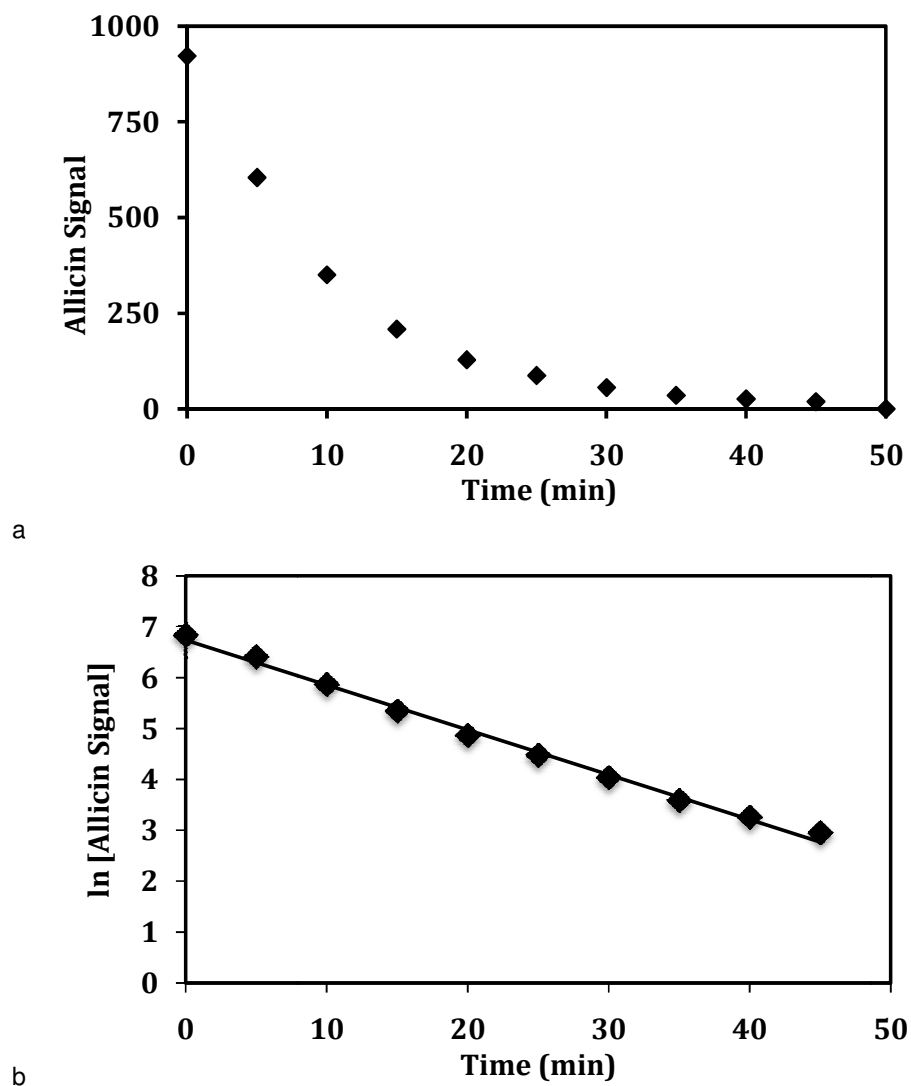
### 3.4 Effect of Incubation Temperature

Aqueous garlic extracts were incubated at other temperatures (4, and 37°C, respectively). 4°C was chosen mainly for the refrigerator storage, while 37°C was chosen because it is the normal body temperature. The allicin degradation process at 4 and 37°C was investigated, respectively as well. As demonstrated in Fig. 3,

the decomposition process was barely observed for allicin in the short period when being incubated at 4°C. However, decomposition did occur over a longer experimental period and the concentration of allicin in the garlic extract decreased gradually and steadily. The allicin signals shown on the graph were the daily average of allicin concentrations ( $n = 6$ ) with the HPLC analyses being performed at the same time on the corresponding experimental days. There was about 15% loss of allicin in the garlic extract after being incubated at 4°C for about a month. When the garlic extract was incubated at 37°C, there was more than 50% loss of allicin over 5-day period. According to the Arrhenius equation,

$$k = Ae^{-\frac{E_a}{RT}} \quad (6)$$

where  $k$  is the reaction rate constant,  $A$  is the pre-exponential factor,  $E_a$  is the activation energy,  $R$  is the gas constant, and  $T$  is the incubation temperature, the reaction rate constant increases with the increasing temperature. [33] The concentration of allicin decreased much faster at 37°C than 4°C, which indicates the degradation rate increases with increasing temperature and verifies the Arrhenius equation prediction. To preserve the therapeutic capabilities of allicin, aqueous garlic extract should be applied as freshly as possible.



**Fig. 2(a).** Detector response changes of allicin in garlic extract when incubated at 100°C over time; **(b).** natural logarithm value of allicin signal changes over time. The HPLC analysis conditions were same as in Fig. 1

### 3.5 Influence of Solvent Media

The concentrated garlic aqueous extracts were diluted with solvents of different polarities to investigate the influence of the solvent media on allicin stability. Water, DMSO, and acetone have different polarities and therefore were used as the solvent for garlic aqueous extract, respectively. The results of solvent effects were shown in Fig. 4. Allicin decomposition occurred at the slowest rate when the garlic extract was diluted in water. The solvents of different polarities give rise to the allicin with distinct nature, affecting the degradation process of

allicin. It has been reported that the allicin decomposition in the different organic medium occurs through different reaction order and theoretical pathways. [25-27] Fujisawa et al also reported that the direct relationship between biological activity and substantial quantity of allicin were observed in ethanolic solvent, hexane and vegetable oil. [25,26] To completely understand the kinetic mechanism of allicin degradation in different solvents, more experiments are still desired. Generally, to take advantage of the therapeutic actions of allicin, solvents should be carefully chosen to dilute the concentrated garlic extracts.

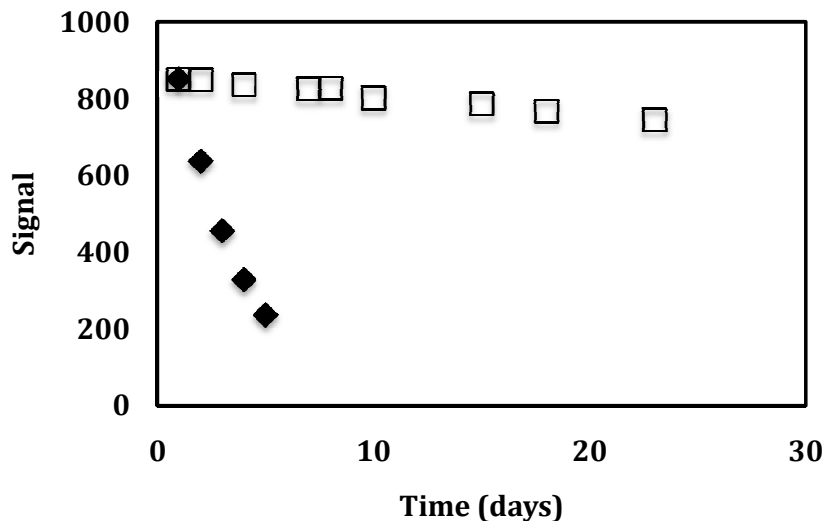


Fig. 3. Degradation of alliin in the garlic extract at different temperatures. The HPLC analysis conditions were same as in Fig. 1. “□” is for the experimental condition of 4°C while “◆” is for 37°C.

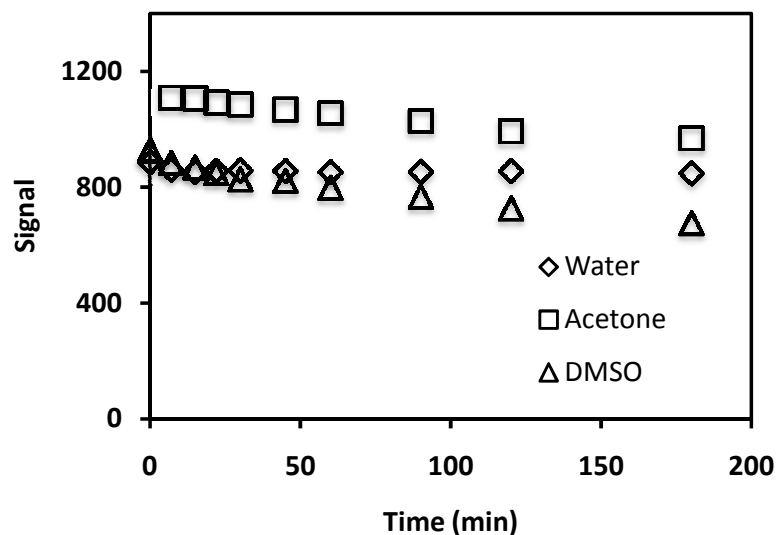


Fig. 4. Degradation of alliin in the garlic extract when incubated in different solvents. The garlic extracts were incubated at 25°C. The HPLC analysis conditions were same as in Fig. 1

#### 4. CONCLUSION

A simple and rapid HPLC-UV method was developed to quantify the amount of alliin in aqueous garlic extracts. The HPLC analysis time of alliin was shortened to within 5 min, compared to traditional 12 min analysis that has been reported in the literature, with no further treatment of garlic extract samples.

Using our methods, the concentration of alliin in the extract samples varied from 35.6 to 44.5 mM. The thermal stability study shows that the degradation of alliin is a first-order reaction and the preliminary data shows that the degradation of alliin in different solvents has different kinetic parameters. To take advantage of therapeutic actions of alliin in garlic extracts, aqueous garlic extract should be applied as freshly as possible to prevent its degradation.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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