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Ultrasonic-assisted extraction of allicin and its stability during storage

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Abstract

Garlic (*Allium sativum* Linn.) has not only been known as culinary purposes but also health and therapeutic benefits. Various important bioactive compounds were found in garlic, especially allicin possessing antifungal and antibacterial activities (Marchese *et al.,* 2016). However, allicin has been claimed to be a heat sensitive compound which may be lost during thermal extraction. Thus, ultrasonic technique was applied for extraction in this study to overcome the problem. The effects of ultrasonic-assisted extraction parameters, i.e., frequency, temperature and time on yield of allicin were investigated. The minimum inhibitory concentration (MIC90) toward *Escherichia coli*, the stability of allicin and kinetic reaction model at different temperatures were also evaluated. The results indicated that higher allicin content exhibited with increase in ultrasonic frequency and then stayed constant after 45 kHz. Extraction temperature of 30˚C provided the higher yield of allicin than that of 20 and 40˚C. Allicin content increased when longer extraction time until reached stable content after extraction time of 40 min. It was also observed that the MIC90 value for inhibiting *E.coli* growth was 12.5 µg/mL. For the stability of allicin in aqueous extract at different temperatures, allicin was more degraded at 40˚C than room temperature (27 ± 2˚C) and 4˚C with half-life periods of 3, 17 and 20 days, respectively. Furthermore, it was found that the kinetic of allicin degradation followed zero order with the highest correlation coefficient (R^2) of 0.95-0.99 and the lowest residual sum of square (RSS) and residual mean of squares (MS) of 0.07-1.51 and 0.01-0.05, respectively.

Keywords: Allicin, Degradation, Extraction, Kinetic, Ultrasonic, Stability

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1. Introduction

Garlic (*Allium Sativum* Linn.) is an herb used as culinary ingredient around the world. It has intensely aromatic and pungent flavor. Garlic is also recognized to a therapeutic medicinal plant since it contains numerous phytochemical compounds which possess anti-tumor and anti-microbial properties (Bayan *et al.,* 2014). Allicin (diallylthiosulfinate), one of the bioactive compounds mostly found in garlic, plays important role in antibacterial effect against a wide range of microorganisms including *Escherichia coli* (Borlinghaus *et al.,* 2014). This compound is heat sensitive and instability (Fujisawa *et al.,* 2014) thus proper extraction treatment of allicin from garlic is a challenge to maintain its quantity and activity.

The conventional extraction of allicin is usually performed using solvent extraction and maceration (Ratti *et al.,* 2007; Rodriguez-Jimenes *et al.,* 2014). These methods are simple and inexpensive. Nevertheless, it requires heating to boiling point of solvent and shaking or stirring during extraction for a long period causing decomposition of allicin (Rafe and Nadjafi, 2014). To overcome the limitations of conventional extraction method, an alternative method should be proposed. Ultrasonic-assisted extraction (UAE) has recently been reported to apply for extraction of bioactive compounds from plants. Ultrasonic wave regarding as high-frequency sound wave above 20 kHz is propagated through liquid media by rarefactions and compression (Danlami *et al.,* 2014). This phenomenon causes formation of bubbles with microjet leading to plant cell breakdown, facilitating the release of bioactive compounds from the plant matrix into liquid media (Vilkhu *et al.,* 2011). It has been proved that UAE has advantages of increase in extraction yield, shorten extraction time, low operation cost and ease of automation (Deng *et al.,* 2017).

UAE efficiency depends on many factors. Hu *et al*. (2016) reported that UAE temperature, processing time and sample/solvent ratio affected anticancer activity and content of polysaccharide from Chuanxiong rhizome. Additionally, González-Centeno *et al.* (2014) revealed that optimal conditions for UAE of total phenolics and total flavonols from grape pomace were found to be 40 kHz whereas Wang *et al.* (2014) reported that 56 kHz was a suitable frequency for UAE of cordycepin from *Cordyceps militaris*. Therefore, the effects of UAE parameters, i.e., frequency, processing time and temperature on yield of allicin were determined in this study. Furthermore, there have been previous reports which studied the ability to against microorganism and stability of allicin extracted using thermal extraction method by determination of the minimum inhibitory concentration (MIC90) and the kinetic parameters for allicin degradation during different storage temperatures (*Yoshida et al.,* 1999; Fujisawa *et al.,* 2008). However, information of the MIC90 and degradation kinetic of allicin

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extracted using UAE are scarce. Thus, the minimum inhibitory concentration (MIC90) toward *Escherichia coli* and the stability of allicin at different temperatures were also investigated.

2. Materials and Methods

2.1 Sample preparation

Thai garlic cloves were obtained from local market (Chiang Mai, Thailand). The transparent shells of garlics were removed manually. The garlic samples were crushed into very small pieces and left in the beaker covered with aluminum foil for 30 min for complete conversion of alliin to allicin by enzyme allinase (Li *et al.,* 2017).

2.2 Conventional extraction

Five grams of the prepared sample was mixed with 100 mL distilled water and centrifuged at 300 rpm (20 x g) for 4 min before filtration through Whatman filter paper No.4. The supernatant was kept in an amber glass bottle at 4˚C for further analysis.

2.3 Ultrasonic-assisted extraction (UAE)

A 600 mL beaker containing a mixture of 5 g of the prepared sample and 100 mL distilled water was placed in an ultrasonic bath (Honda electronics, W 113, Japan). The UAE was carried out at frequencies of 28, 45 and 100 kHz, extraction temperatures of 20, 30 and 40˚C and extraction times of 20, 40 and 60 min. At the end of extraction, the suspension was filtered through Whatman no. 4 filter paper to remove solid debris. The supernatant was then kept in an amber glass bottle at 4˚C for further analysis.

2.4 Determination of allicin content

Allicin content in garlic extracts were determined using the method described by Miron *et al.* (2002) 0.2 mL of each allicin extracts was placed in a test tube containing 4 mL of 4-Mercaptopyridine solution; 20 mg of 4-Mercaptopyridine was dissolved in 2 mL 50% ethanol and diluted with 50 mM Na-phosphate, 2 mM EDTA, pH 7.2 (buffer A). The mixture solution was stirred at room temperature for 30 min. Decrease in optical density (OD) of the mixture solution was determined using spectrophotometer (Shimadzu, UV21101 PC, Kyoto, Japan) at 324 nm. The concentration of allicin was evaluated using equation (1).

$$[A] = \Delta A_{324} \times 0.499 \qquad (1)$$

where [*A*] is concentration of allicin (mg/mL)

ΔA_{324} is A_{324} (4-MP without allicin) – A_{324} (4-MP with allicin)

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2.5 Determination of Minimum Inhibitory Concentration (MIC90)

Minimum inhibitory concentration (MIC90) was performed using broth dilution technique (Seema *et al.,* 2015). Briefly, 12 sterile 7.5 x 1.3 cm capped tubes were prepared in a rack. A 1 mL of nutrient broth was added into all the capped tubes except the first capped tube. After that, garlic extracts with concentration of 100 µL/mL in sterile nutrient broth were pipetted into the second capped tube. The 1 mL of a mixture solution in the second capped tube was then transferred to the third capped tube using a fresh pipette and continued preparing dilutions in this way until the 11^{th} capped tube. The last tube was free of garlic extracts used as a control. A 1 mL of nutrient broth containing *E.coli* culture of 10^6–10^7 cell/ml was pipetted into all capped tubes and incubated further for 24 h at 37°C to serve for growth or turbidity. The turbidity was determined spectrophotometrically at 540 nm. MIC90 was expressed as the lowest dilution which percent inhibited growth judged by lack of turbidity in the tube. The percent inhibited growth can be calculated by equation (2). The experiments were repeated three times.

$$\%\ Inhibition = [(Abs_{control} - Abs_{sample})/Abs_{control}] \times 100 \qquad (2)$$

where $Abs_{control}$ is absorbance of control

Abs_{sample} is absorbance of sample

2.6 Reaction order of allicin during different storage temperatures

Allicin extracts were stored at 4 ± 1˚C, 27 ± 2˚C and 40 ± 1˚C. The degradation of allicin in aqueous solution during storage was determined using zero-order (equation (3)), first–order (equation (4)) and second-order (equation (5)) kinetic models (van Boekel, 2008). The most appropriate model was selected based on the correlation coefficients (R^2), residual sum of squares (RSS) and residual mean of squares (MS) calculated using the least square procedure.

$$[A] = [A]_0 - k_1 t \qquad (3)$$

$$\ln[A] = \ln[A]_0 - k_2 t \qquad (4)$$

$$\frac{1}{[A]} = \frac{1}{[A]_0} + k_3 t \qquad (5)$$

where A is the allicin concentration (mg/mL) at time t

A_0 is the allicin concentration (mg/mL) at time 0

k_0, k_1 and k_2 are the allicin degradation rate constant for the zero order ((mg/ml).day^{-1}), for the first order (day^{-1}) and for the second order ((ml/mg).day^{-1}), respectively

t is the storage time (days)

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Half-life ($t_{1/2}$) of allicin is the estimated time where the concentration of allicin is decreased by 50% from its initial value ($A = 0.5A_0$). Half-life of each allicin sample at its corresponding storage temperature is determined using the equation (6) (zero order) or equation (7) (first order) or equation (8) (second order) depending on the best fitted model to the experimental data.

$$t_{1/2} = \frac{[A]_0}{2k_0} \quad (6)$$

$$t_{1/2} = \frac{\ln 2}{k_1} \quad (7)$$

$$t_{1/2} = \frac{1}{k_2[A]_0} \quad (8)$$

where $t_{1/2}$ is the half-life of reaction

A_0 is the allicin concentration (mg/ml) at time 0

k is the allicin degradation rate constant

2.7 Data analysis and statistical analysis

The experiments were designed to be completely random. The presented results are mean of experimental values with standard deviations. The analysis of variance technique and Tukey's multiple range tests were used to determine the significant difference in allicin yield of different extraction treatments at 95% confidence level ($P<0.05$). All experiments were performed in triplicate unless specified otherwise.

3. Results and discussion

3.1 Yield of allicin at different UAE conditions

Allicin concentration of garlic extracts during UAE with three different frequencies (28, 45 and 100 kHz) and three different extraction temperatures (20, 30 and 40˚C) was determined and the results are shown in Fig 1a–c. It was observed that longer extraction time gave higher allicin content at ultrasonic frequency of 28 kHz. However, during UAE at frequency of 45 and 100 kHz allicin content increased initially and then decreased slightly at 60 min. This might be because ultrasonic technique related to mechanical agitation effect and cavitation effect on plant cells causing cell disruption and subsequently facilitated the release of the compounds from plant matrix (Tomsik *et al.,* 2016). Nevertheless, at high frequency allicin could also be degraded by the mechanical agitation effect and cavitation effect when extraction time was prolonged. The results were consistent with previous reports (Liu *et al.,* 2017). For example, Liu *et al.* (2017) revealed that yield of phenolic compounds from *Phyllanthus urinaria* first increased and then gradually decreased when longer ultrasonic time during UAE at 53 kHz. Moreover, the results showed that UAE at 40 min provided the highest allicin content.

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For the effects of extraction temperatures at 20, 30 and 40˚C on yield of allicin, it was found that allicin content increased with the higher temperature. However, the allicin content was reduced when extraction temperature was over 30˚C. Since higher temperature helped break down plant cell, bioactive compounds might be more leached into solvent. Spigno *et al.* (2007) stated that yield of phenolic compounds extracted from grape at 60˚C was higher than that at 45˚C. Nonetheless, in earlier studies, reported that allicin could be degraded at temperature above 36˚C. Hence, reduction of allicin content at UAE temperature of 40˚C could occur.

When determining the changes of allicin content during UAE at 25, 45 and 100 kHz, it was evident that allicin content increased with the increase in the ultrasonic frequency up to 45 kHz and then allicin content stayed constant when the ultrasonic frequency was increased to 100 kHz. This was probably because of the mechanical and cavitation effects. Since cavitation is associated with a formation of bubbles in a liquid to create high-velocity inter-particle collisions and turbulence leading to breakdown of plant cell, size and characteristic of bubbles depend on ultrasonic frequency. At too low frequency, the generated bubbles in solution are large and unstable which easily collapse to generate high localized temperature and pressure jets. Nevertheless, time interval between compression and rarefaction in compression cycle is longer resulting in slowly occurring cavitation. Under ultrasonic treatment at too high frequency, the generated bubbles in solution are smaller and more stable leading to weaker cavitation intensity. The obvious cavitation effect is not then obtained by the collapse bubbles (Zhang *et al.,* 2017; Garcia−Vaquero *et al.,* 2018). Based on the results, ultrasonic frequency at 45 kHz was suitable for extraction of allicin from garlic. The similar result has been shown in previous studies. for example, reported that UAE of lycopene from tomatoes at frequency of 48 kHz exhibited the highest extraction efficiency.

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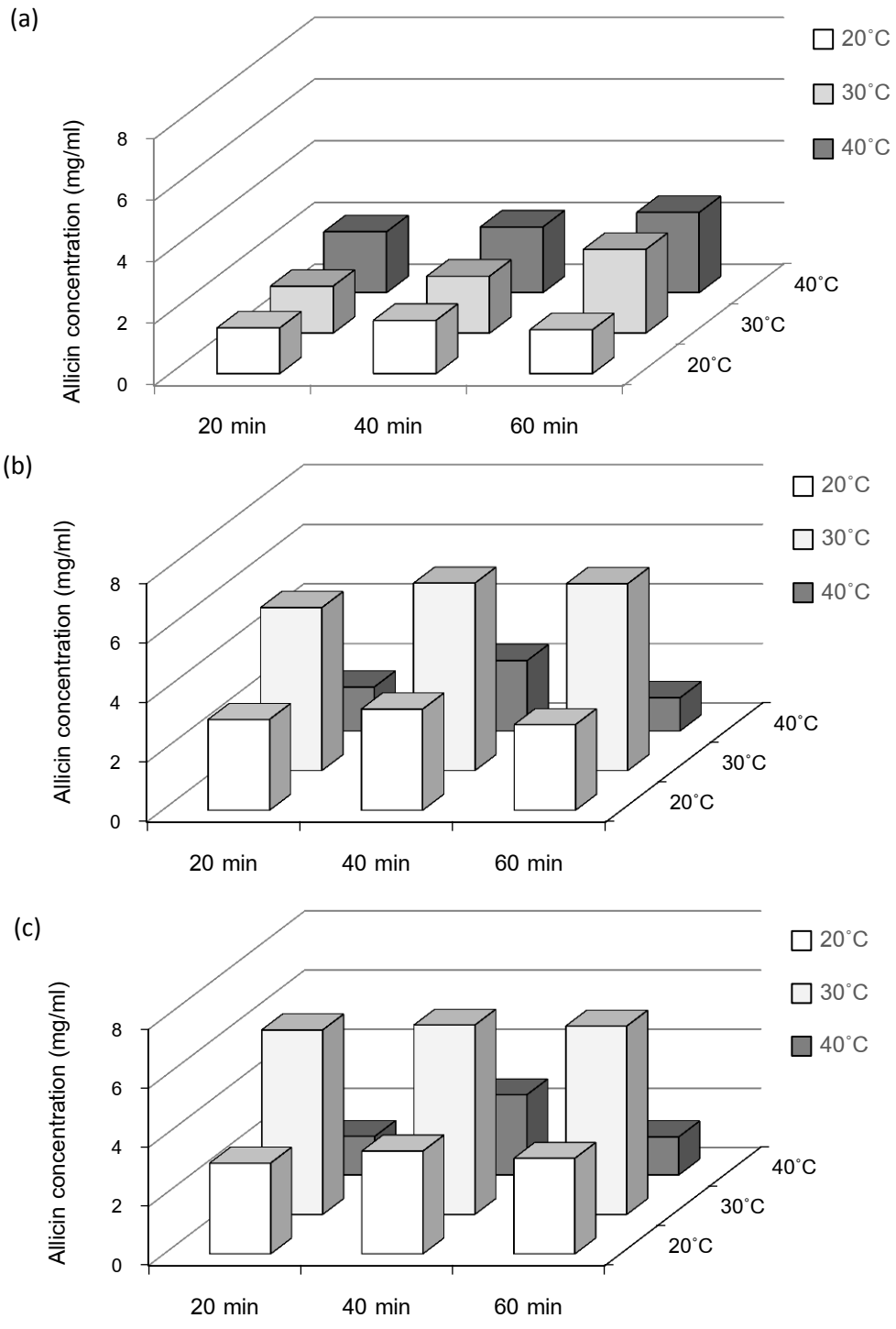

Fig 1 Allicin concentration obtained by UAE for (a) 28 kHz (b) 45 kHz and (c) 100 kHz at different extraction times and temperatures

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3.2 Comparison between conventional extraction and UAE

The UAE condition of 45 kHz, 30˚C for 40 min was performed as it was the optimal extraction condition. Yields of allicin obtained by conventional extraction and UAE were compared and shown in Fig 2. It was observed that UAE exhibited significantly higher yield of allicin than the conventional extraction. Ultrasonic treatment could improve extraction yield by the mechanical and cavitation effects as mentioned above. The plant cell wall would be effectively destroyed by ultrasound waves which facilitate the leaching of bioactive components into the solvent. Moreover, ultrasonic enhanced molecular motion of the solvent leading to quickly combining the bioactive components with the solvent (Zhang *et al.,* 2017). However, conventional extraction involved only mechanical agitation effect so that it tended to be less extracted yield when compared to UAE. The results were in agreement with Zlabur *et al.* (2016) who reported that higher content of total carotenoids in lemon balm and peppermint were obtained with UAE when compared to conventional method.

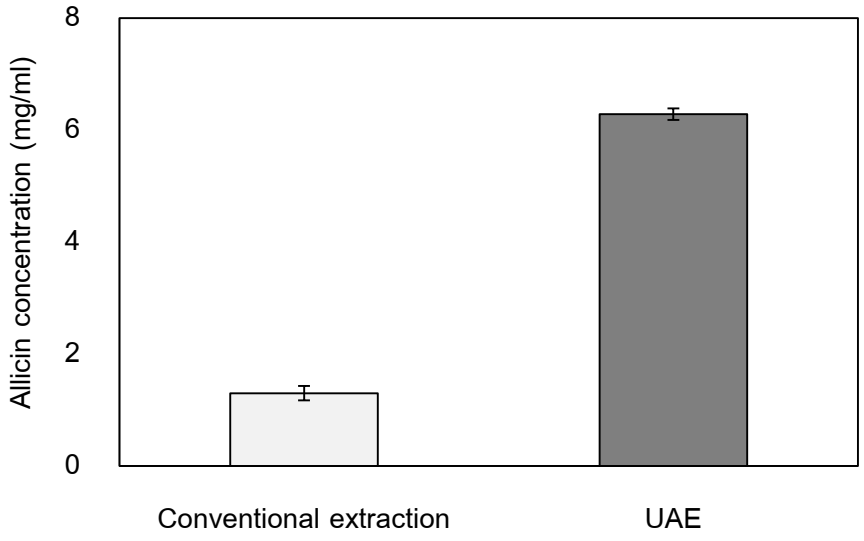

Fig 2 Allicin concentration after conventional extraction and UAE

3.3 Determination of Minimum Inhibitory Concentration (MIC90)

To determine MIC90 value, percent inhibition of the *E.coli* growth at different allicin concentrations after overnight incubation were determined and presented in Table 1. MIC90 value was indicated as the lowest allicin concentration when percent inhibition of *E.coli* growth was higher than 90%. The results showed that allicin concentration of 12.50 µg/mL exhibited the lowest concentration against *E.coli* with percent inhibition above 90%. This observation value was in a similar range to Yoshida *et al.* (1999) who reported that MIC90 of *E.coli* growth by allicin derived from garlic was 15 µg/mL.

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Table 1 Percent inhibition of *E.coli* growth at different allicin concentrations

Allicin concentration (µg/mL)	Inhibition (%)
50.00	99.30 ± 0.31[a]
25.00	98.75 ± 0.23[ab]
12.50	97.75 ± 0.53[b]
6.25	56.44 ± 3.17[c]
3.13	27.25 ± 15.24[d]
1.56	7.79 ± 1.48[e]

Same letters in the same column indicate that the values are not significantly different (P>0.05).

3.4 Kinetics of allicin during different storage temperatures

The allicin extracted by UAE at 45 Hz and 30˚C for 40 min was stored at 4, 27 and 40˚C. Changes of allicin concentration during storage were presented in Fig 3. The results showed that allicin concentrations continuously decreased when storage time were longer for all storage temperatures. It was also observed that reduction rate of allicin concentration at 40˚C was faster than 4 and 27˚C. This was probably because decomposition of allicin could occur by either two mechanisms which were self−decomposition and thermolysis (Dongsheng *et al.*, 2014; Wang *et al.,* 2015). Allicin is an unstable, odorous compound that decomposes to form an array of second-generation sulfur-containing compounds, such as allyl sulfides and allyl polysulfides so that prolong storage time causes more reduction of allicin content (Wang *et al.,* 2015). Moreover, allicin compound is sensitive to heat which appears degradation at temperature above 36˚C, as mentioned above (Dongsheng *et al.,* 2014). Therefore, there was only self−decomposition of allicin at 4 and 30˚C whereas there were 2 decomposition mechanisms of self−decomposition and thermolysis at 40˚C (Dongsheng *et al.,* 2014). This resulted in larger degradation at storage temperature of 40˚C than 4 and 30˚C.

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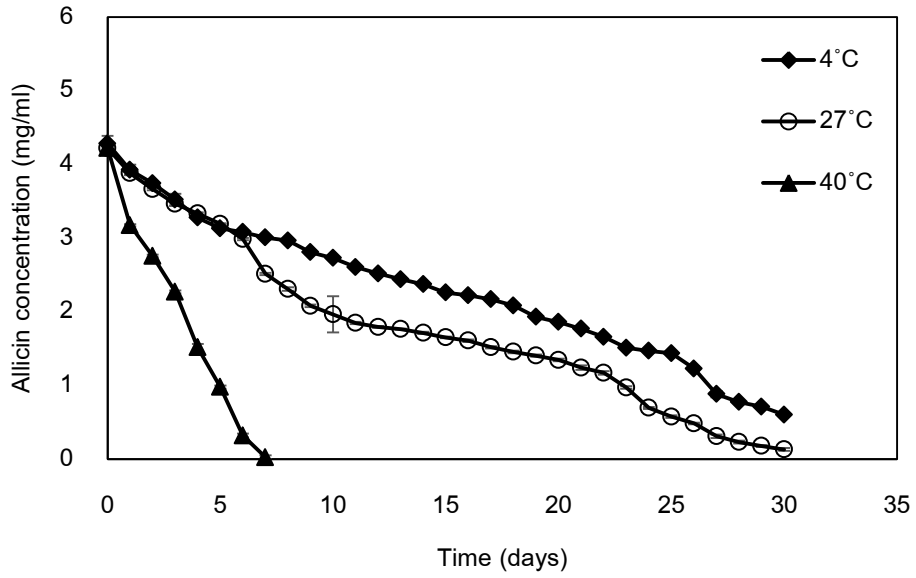

Fig 3 Changes of allicin concentration during storage at different temperatures

Experimental data during storage were analysed for degradation kinetics with different orders. Fig 4 presented the fitted experimental data with zero–order, first–order and second–order equations. It can be seen that the experimental data for all storage temperatures were more fitted to the zero order model than the first and second order models. The highest coefficient of determination (R^2) and the lowest residual sum of square (RSS) and residual mean of squares (MS) presenting in Table 2 also were exhibited with the zero–order model for all cases. This was agreement with results obtained by Phoungchandang and Boonnattakorn (2008). Their study reported that allicin decomposition during storage at 30˚C followed zero order reaction with a high correlation coefficient (R^2) of 0.99.

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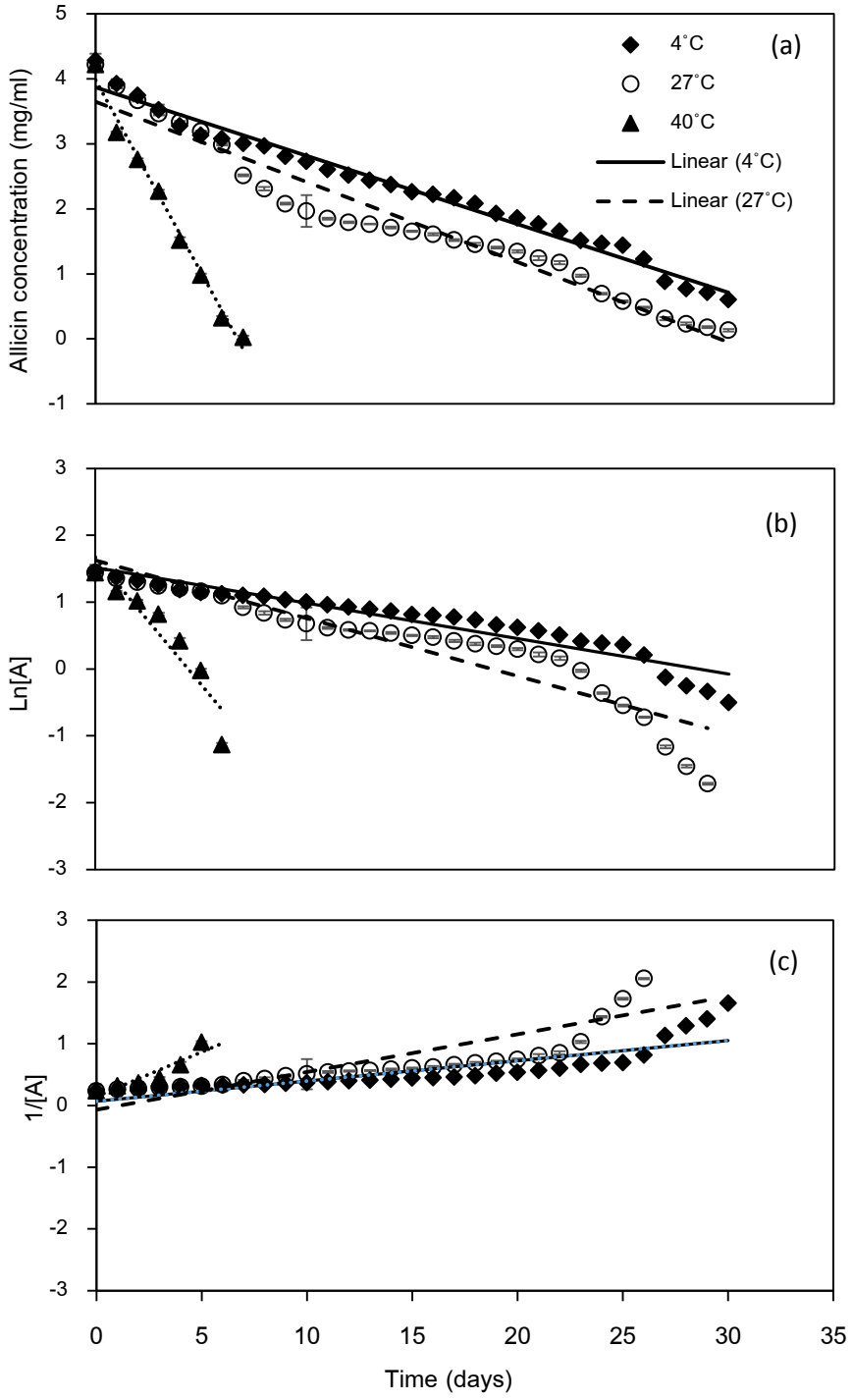

Fig 4 Experimental data fitted with (a) zero order, (b) first order and (c) second order kinetic model

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Table 2 Constant rate and coefficient of determination (R^2), residual sum of squares (RSS) and residual mean of squares (MS) of zero, first and second order models

Kinetic order	Temperature (˚C)	Constant rate (k)	R^2	RSS	MS
Zero order	4	-0.105	0.98	0.34	0.01
	27	-0.123	0.95	1.51	0.05
	40	-0.592	0.99	0.07	0.01
First order	4	-0.053	0.90	0.53	0.02
	27	-0.093	0.86	2.50	0.09
	40	-0.602	0.87	0.45	0.11
Second order	4	0.033	0.71	0.67	0.02
	27	0.132	0.60	4.35	0.17
	40	3.370	0.87	0.04	0.01

Since allicin degradation followed the zero order model, half–life of allicin at different storage temperatures could be calculated by equation (6). As shown in Table 3, it was obvious that storage temperature of 4˚C exhibited longer half–life time than that of 30 and 40˚C because thermal degradation of allicin hardly occurred. Similar result was found to Fujisawa *et al.* (2008) who revealed that half–life value of allicin at 4˚C was larger than 15 and 37˚C.

Table 3 Half–life of allicin at different storage temperatures

Temperature (˚C)	Half–life period (days)
4	20.38
27	17.40
40	3.61

4. Conclusions

Ultrasonic technology can be applied to extract allicin substance from garlic. The UAE parameters which were frequency, extraction temperature and time on the yield of allicin were determined to obtain the best UAE condition. A comparison between the yield of allicin from conventional extraction and UAE was also investigated. The results showed that UAE at frequency of 45 kHz and 30˚C for 40 min was recommended since it provided the highest yield of allicin. UAE gave 4.8 times greater in the yield of allicin than conventional method. Moreover, the MIC90 of allicin against *E.coli* and its stability during storage at 4, 27 and 40˚C were investigated. It was found that larger degradation of allicin occurred at higher storage

temperature. Half-life period of allicin at 4, 27 and 40˚C were 20, 17 and 3 days, respectively. The kinetic of allicin degradation followed the zero order model with the highest R^2 and the lowest RSS and MS values.

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