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Salting-out extraction of allicin from garlic (*Allium sativum* L.) based on ethanol/ammonium sulfate in laboratory and pilot scale

Fenfang Li [b], Qiao Li [b], Shuanggen Wu [b], Zhijian Tan [a,*]

[a] Institute of Bast Fiber Crops and Center of Southern Economic Crops, Chinese Academy of Agricultural Sciences, Changsha 410205, China
[b] College of Chemistry and Chemical Engineering, Central South University, Changsha 410083, China

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ABSTRACT

Salting-out extraction (SOE) based on lower molecular organic solvent and inorganic salt was considered as a good substitute for conventional polymers aqueous two-phase extraction (ATPE) used for the extraction of some bioactive compounds from natural plants resources. In this study, the ethanol/ammonium sulfate was screened as the optimal SOE system for the extraction and preliminary purification of allicin from garlic. Response surface methodology (RSM) was developed to optimize the major conditions. The maximum extraction efficiency of 94.17% was obtained at the optimized conditions for routine use: 23% (w/w) ethanol concentration and 24% (w/w) salt concentration, 31 g/L loaded sample at 25 °C with pH being not adjusted. The extraction efficiency had no obvious decrease after amplification of the extraction. This ethanol/ammonium sulfate SOE is much simpler, cheaper, and effective, which has the potentiality of scale-up production for the extraction and purification of other compounds from plant resources.

1. Introduction

Garlic (*Allium sativum* L.) is a well-known edible and medicinal plant since ancient China. Allicin (diallylthiosulfinate) is an organosulfur compound, and it is one major biological active substance in garlic (Tyagi, Pradhan, Srivastava, & Mehrotra, 2014). Actually, allicin is converted from alliin after crushing of the garlic clove under the action of alliinase (Amagase, Petesch, Matsuura, Kasuga, & Itakura, 2001; Ankri & Mirelman, 1999). Allicin was first studied for its antibacterial properties in the middle of 20th century (Cavallito & Bailey, 1944), then its other pharmacological actions of anti-oxidant, antifungal, antihypertensive, anti-inflammatory, and inhibition of tumor were also found (El-Kashef, El-Kenawi, Suddek, & Salem, 2015; Hirsch et al., 2000; Liu, Ren, Wang, Yao, & He, 2015). Up to now, the solvent extraction using water or ethanol aqueous solution (Arzanlou & Bohlooli, 2010; Bocchini, Andalo, Pozzi, Galletti, & Antonelli, 2001; Wang et al., 2014) and supercritical fluid extraction (SFE) (del Valle, Glatzel, & Martinez, 2012; Liang, Qiao, Bi, Zou, & Zheng, 2012; Rybak, Calvey, & Harnly, 2004) are the most widely used methods for the extraction of allicin from garlic in laboratory, pilot or large scale. However, the solvent extraction can obtain the crude extract and the samples need further purification; SFE requires sophisticated instrument and high cost.

Aqueous two-phase extraction (ATPE) was first introduced by Albersson, and the most commonly used two aqueous two-phase systems (ATPSs) were PEG/salt and PEG/dextran (Albertsson, 1986). ATPS based on low molecular organic solvents (e.g. methanol, ethanol, acetone, and n-propanol) and inorganic salts had been developed in recent years, which can also be called salting-out extraction (SOE) system (Dong et al., 2016). Compared with polymer ATPS, SOE has the advantages of lower cost, lower viscosity, quicker phase separation time, relatively lower environmental toxicity and easier to scale up (Amid, Shuhaimi, Sarker, & Manap, 2012; Fu, Yang, & Xiu, 2015; Liu, Zou, Gao, Gu, & Xiao, 2014; Ooi et al., 2009; Wang, Han, Xu, Hu, & Yan, 2010). SOE systems had been used to extract various bioactive compounds from different plants resources, such as anthocyanins from grape juice (Wu et al., 2014), alkaloids from *Sophora flavescens* Ait. (Zhang et al., 2015), phenolic compounds from *Ficus carica* L. (Feng et al., 2015), rutin from acerola waste (Reis et al., 2014), lignans from *Zanthoxylum armatum* (Guo, Su, Huang, Wang, & Li, 2015), and polysaccharides from *Semen Cassiae* (Chen et al., 2016).

The objective of this study is to use SOE for the extraction and preliminary purification of allicin from garlic powder. The allicin is extracted into alcohol-rich phase, while partial impurities are extracted into the salt-rich phase. The extraction conditions were

* Corresponding author.
E-mail address: tanzhijiantgzy2010@aliyun.com (Z. Tan).

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optimized at laboratory scale, SOE was scaled up to the pilot scale under the optimized conditions. The phase-forming components of ethanol and ammonium sulfate were recycled and reused.

2. Materials and methods

2.1. Materials and reagents

The garlic samples were originated in Shandong province and bought from the Vanguard supermarket in Changsha City, Hunan Province. The allicin standard was purchased from National Institutes for Food and Drug Control (Beijing, China) with HPLC purity larger than 98%. HPLC grade acetonitrile was purchased from TEDIA Company, Inc. (Fairfield, OH, USA). The analytical reagents of different organic solvents (ethanol, n-proanol, isopropanol, acetone and acetonitrile) and salts (ammonium sulfate, sodium dihydrogen phosphate, sodium sulfate, and potassium phosphate) were provided by Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). 2,2-diphenyl-1-picpicrylhydrazyl (DPPH), ferrous sulfate, salicylic acid hydrogen peroxide were purchased from Alladin Reagent Co., Ltd. (Shanghai, China). *Escherichia Coli* DH5α was provided by Tiangen Biotech Co., Ltd. (Beijing, China). All other reagents used in this study were analytical grade and no further treatments were processed for them.

2.2. Preparation of crude extract

The garlic was peeled, then smashed into powder. In order to make complete converting of alliin to allicin, a grinding time of 30 min was employed. Then the garlic powder was extracted by absolute ethanol with ultrasonic assisted extraction for 20 min in an ultrasonic bath (model KQ-5200 DE, Kunshan Ultrasound Co. Ltd., Kunshan, China). The mixture was centrifugated and filtrated to remove the insoluble substance. The supernate was evaporated to remove the ethanol, the crude extract of allicin was obtained.

2.3. Phase diagram

The phase diagrams indicate the information of the concentration of each component is required to form the ATPS, which were drawn by the similar turbidimetric titration reported in our previous work (Tan, Li, & Xu, 2013). Firstly, organic solvent of known mass was added into a tube. Then, a salt solution of known mass fraction was added dropwise, and the solution in the tube was well mixed. The solution became turbid after adding of the salt solution then separated into two phases. The mass fraction of each added component was calculated. Lastly, a few drops of water was added to make the mixture clear again. The above procedures were repeated to obtain sufficient data to construct the phase diagrams.

2.4. Salting-out extraction

SOE system was formed by adding salt aqueous solution into a tube containing a certain amount of organic solvents, then crude allicin extract was added. ATPS was used for the SOE of allicin after complete stirring. Allicin was extracted into the top phase (organic solvent-rich phase), while some hydrophilic substances, such as alliin, lysine, glutamic, *etc*., tended to partition in the bottom phase (salt-rich phase) (Jiang, Lu, Tan, & Cui, 2014). Centrifugation was performed to accelerate the phase-forming and achieve complete extraction, then two clear phases formed and the volume of each phase was noted down. HPLC analysis was used for the quantitative determination of the allicin concentration. The extraction efficiency (E/%) of allicin in top phase was calculated by the Eq. (1).

$$E = \frac{C_t V_t}{C_o v_o} \times 100\% \quad (1)$$

where C_t was the allicin concentration in top phase, and V_t was the volume of top phase, C_o and V_o were the concentration and volume, respectively of crude allicin extract added into the system.

2.5. HPLC conditions

The allicin samples were analyzed using a Dionex UltiMate 3000 HPLC system (Dionex, Sunnyvale, California, USA) coupled with a LPG-3400 pump, a VWD-3400 UV–vis detector, and a TCC-3000 column compartment. A Promosil C_{18} chromatographic column (250 × 4.6 mm i.d., 5 μm particle size) was used to analyze the samples. A PC coupled with a Chameleon software was used to collect and analyze the data. The mobile phase was composed of acetonitrile, H_2O, and acetic acid (75:25:0.6; *v*/*v*/*v*). The flow rate was 1.0 mL/min with isocratic elution and the effluent was monitored at the wavelength of 240 nm. The oven temperature was set at 30 °C. All the samples of 20 μL were injected into the HPLC for analysis. The mobile phase and samples solution were filtered through microfiltration membrane (0.45 μm) before analysis. The standard curve for analysis of allicin is $A = 292.67C + 0.34$ ($R^2 = 0.9995$), where A is the peak area and C is the allicin concentration. Standard solutions of allicin were diluted in the range from 0.024 to 0.114 mg/mL.

2.6. Antioxidant activity of allicin obtained by SOE

The free-radical scavenging activity of allicin was assessed using DPPH· radical according to the method with moderate modification (Athukorala, Kim, & Jeon, 2006). Two milliliter allicin solution was added into 2.0 mL DPPH· ethanol solution (40 μg/mL). Another two samples were prepared with 2.0 mL ethanol being added into 2.0 mL DPPH· (as blank) and 2.0 mL allicin concentration solution being added into 2.0 mL ethanol (as control), respectively. The mixture was shaken vigorously and left to stand for 40 min at ambient temperature in darkness. Absorbance was determined at 517 nm by a UV–Vis spectrophotometer (UV-2100, Unico, USA), vitamin c (V_c) was used as a comparison to allicin. The results were expressed as percentage inhibition of the DPPH· radical on Eq. (2).

$$\text{Scavenging capacity } (\%) = [1 - (A_s - A_c)/A_o] \quad (2)$$

where A_c, A_s and A_o represent the absorbance of control, sample and blank, respectively. The antioxidant capacity of test compounds was expressed as IC_{50}, the concentration necessary for 50% reduction of DPPH·.

The scavenging ability of hydroxyl radical (·OH) was evaluated according to the modified Fenton method (Wang & Jiao, 2000). To a colorimetric tube, 4.0 mmol/L $FeSO_4$, 10.0 mmol/L H_2O_2, 5.0 mmol/L salicylic acid ethanol solution and a certain concentration of allicin sample were added. The proportion of each component was indicated as follow: A_c (1.0 mL $FeSO_4$ + 0.5 mL salicylic acid + 7.5 mL H_2O + 1.0 mL H_2O_2), A_s (1.0 mL $FeSO_4$ + 0.5 mL salicylic acid + 7.5 mL allicin sample + 1.0 mL H_2O_2), and A_o (1.0 mL $FeSO_4$ + 0.5 mL H_2O + 7.5 mL allicin sample + 1.0 mL H_2O_2). Each mixture was vigorously shaken and left to stay for 30 min at ambient temperature in darkness. The absorbance was determined at 510 nm by a UV–Vis spectrophotometer (UV-2100, Unico, USA) and the scavenging radical activity was calculated according to the identical equation (5) used in DPPH· determination. The antioxidant capacity of test compounds was expressed as IC_{50}, the concentration necessary for 50% reduction of ·OH.