

## Three-liquid-phase Extraction and Separation of Capsanthin and Capsaicin from *Capsicum annum* L.

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### Abstract

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The extraction of capsanthin and capsaicin from red pepper (*Capsicum annum* L.) was studied using a three-liquid-phase system (TLPS) of acetone/K<sub>2</sub>HPO<sub>4</sub>/*n*-hexane. When the system consisted of 22% (w/w) acetone/20% (w/w) K<sub>2</sub>HPO<sub>4</sub>/10% (w/w) *n*-hexane, capsanthin was extracted into the top *n*-hexane-rich phase, yielding a recovery of 98.15% at a temperature of 25°C. Meanwhile, capsaicin was mainly distributed in the middle acetone-rich phase, less than 0.01% in the top phase, and undetectable in the bottom salt-rich phase. The yields of capsanthin and capsaicin were 105 and 88% of those of the conventional solvent extraction, respectively. Thus, capsanthin and capsaicin were separated through a single step at a low cost.

**Keywords:** partition behaviour; red pepper; recovery; three-liquid-phase system (TLPS)

Natural effective components extracted from plants have received considerable attention because of their physiological functions and safety. Capsanthin is the most important carotenoid pigment in red pepper (KIM *et al.* 2002). It has different properties such as antioxidant and immunoenhancing activities (RHIM *et al.* 2011; TOPUZ *et al.* 2011). Capsaicin (8-methyl-*N*-vanillyl-*trans*-6-nonenamide) is the main pungency compound isolated from red pepper (OTHMAN *et al.* 2011). As a spicy by-product, it affects the quality of capsanthin extracted from hot red pepper. In fact, capsaicin is also used to treat pain and inflammation associated with various diseases (SHAKHIDOYATOV *et al.* 2001; ZHANG *et al.* 2008; KIM *et al.* 2009).

Capsanthin and capsaicin are usually extracted using conventional organic solvents and then isolated from the crude extract. This multistep process has some disadvantages such as high consumption of energy, inefficiency, and need of large amounts of a solvent. Although supercritical carbon dioxide (SC-CO<sub>2</sub>) extraction, a new method, can be used to extract capsanthin and capsaicin (UQUICHE *et*

*al.* 2004; KWON *et al.* 2011), it has no advantage in the extraction of some polar components, such as capsanthin and capsaicin, because carbon dioxide is a weak polar solvent. In addition, SC-CO<sub>2</sub> extraction has stringent equipment requirements.

The salting-out extraction (SOE) system composed of short-chain alcohol, hydrophobic organic solvents, inorganic salt, and water is a novel method used to separate active constituents from fermentation broth or natural plant (LIU *et al.* 2010; DAI *et al.* 2011). Recently, aqueous two-phase system (ATPS) and three-liquid-phase system (TLPS) have been used to extract and isolate effective components from natural products such as anthocyanins from mulberry (WU *et al.* 2011), geniposide from gardenia fruit (PAN *et al.* 2002), piceid, resveratrol, and emodine from *Polygonum cuspidatum* (WANG *et al.* 2008), and salvianolic acid B from the crude extract of *Salvia miltiorrhiza* (Zhi *et al.* 2006). This method has many advantages such as low solvent requirement, low cost, low equipment requirements, and short extraction time.

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This study aims to establish TLPS with hydrophilic and hydrophobic organic solvents and inorganic salt aqueous solution as well as to extract capsanthin and capsaicin from red pepper in a single step. A fast extraction and separation method of capsanthin and capsaicin was realised by analysing the distribution of capsanthin and capsaicin in different phases.

## MATERIAL AND METHODS

**Material.** Mature hot red pepper purchased from Changning (Sichuan Province, China) was ground into powder (80–300  $\mu\text{m}$ ) by a miniature high-speed universal pulveriser (FW80; Suzhou Jiangdong Precision Instruments Co., Ltd., Suzhou, China). Paprika is the powder form of dry red pepper. Capsaicin (98% purity) was purchased from the National Institute for the Control of Biological and Pharmaceutical Drugs of China (Beijing, China). All other reagents are of analytical grade.

**Analytical methods.** Total capsanthin pigment content was measured using a spectrophotometric method. The extracted oleoresin was dissolved in 100 ml of acetone. 1 ml was diluted to a suitable concentration with acetone. Absorbance was obtained at 460 nm using a Jasco V-560 UV/Vis spectrophotometer (Jasco Analytical Instruments, Tokyo, Japan). Capsanthin concentration was calculated according to the method described by JARÉN-GALÁN *et al.* (1999). To calculate the yield of extracted pigment, the capsanthin obtained by lixiviation in 95% acetone was adopted as a yield of 100%.

Capsaicin was determined by high-performance liquid chromatography (HPLC) using RP-C<sub>18</sub> column (5  $\mu\text{m}$ , 4.6 mm  $\times$  250 mm) (both Agilent Technologies, Inc., Santa Clara, USA). A mobile phase consisting of water, 0.1% phosphoric acid (A) and methanol (B) at a volume ratio of 30:70 was eluted at a flow rate of 1.0/minute. Capsaicin was monitored at 290 nm. Column department temperature was 30°C. Injection volume and run time were 20  $\mu\text{l}$  and 25 min, respectively.

**Aqueous two-phase extraction of capsanthin and capsaicin from red pepper.** Predetermined quantities of acetone, K<sub>2</sub>HPO<sub>4</sub> and water were weighed and added to paprika (mass ratio = 1:25). The mixture was thoroughly vibrated for 10 min and then settled at room temperature. After the separation of two phases, the volumes of the acetone and water phases of the system were recorded, and capsaicin and capsanthin were analysed.

Subsequently, 1 ml of the acetone or water phase was added to two volumes of anhydrous methanol, mixed

by vortexing, and then centrifuged at 12 000 rpm for 20 minutes. The supernatant was filtered into a 2-ml glass vial using a 0.45  $\mu\text{m}$  membrane filter (Millipore, Shanghai Xihe Technology Co., Ltd., Shanghai, China) and used for HPLC injections.

The partition coefficient (*K*) of capsanthin/capsaicin was defined as the concentration ratio of capsanthin/capsaicin in the acetone phase to that in the water phase. The recovery (*R*, %) of capsanthin/capsaicin was defined as the mass ratio of capsanthin/capsaicin in the acetone phase to its total amount in ATPS.

**Three-liquid-phase extraction of capsanthin and capsaicin from red pepper.** Based on ATPS consisting of acetone/K<sub>2</sub>HPO<sub>4</sub>/water, *n*-hexane or petroleum ether was added to establish TLPS. The mixture of paprika and TLPS components was vigorously mixed and then settled at room temperature for phase separation. The volumes of the top, middle, and bottom phases of the system were noted, and capsaicin and capsanthin were analysed.

Different extraction procedures: (A) acetone and *n*-hexane were vigorously mixed with paprika for 10 min, and then K<sub>2</sub>HPO<sub>4</sub> solution was added; (B) acetone and K<sub>2</sub>HPO<sub>4</sub> solution were vigorously mixed with paprika for 10 min, and then *n*-hexane was added; (C) K<sub>2</sub>HPO<sub>4</sub> solution and *n*-hexane were vigorously mixed with paprika for 10 min, and then acetone was added; (D) the mixture of K<sub>2</sub>HPO<sub>4</sub> solution, acetone, and *n*-hexane was vigorously mixed with paprika for 10 minutes.

The *n*-hexane/petroleum phase (1 ml) was evaporated to remove *n*-hexane/petroleum ether, and the residue was dissolved in an equal volume of anhydrous methanol for capsaicin analysis in the top phase. Capsaicin in the acetone phase was measured similarly like in ATPS.

The partition coefficient (*K*) of capsanthin was defined as the ratio of the concentration in the *n*-hexane/petroleum phase to that in the acetone phase. The recovery (*R*, %) of capsanthin was defined as the mass ratio partitioned in the *n*-hexane/petroleum phase to its total amount. The partition coefficient (*K*) of capsaicin was defined as the ratio of the concentration in the acetone phase to that in the *n*-hexane/petroleum phase. The recovery (*R*, %) of capsaicin was defined as the mass ratio partitioned in the acetone phase to its total amount. The phase ratio was expressed as the ratio of the volume of the *n*-hexane/petroleum phase to that of the acetone phase.

## RESULTS AND DISCUSSION

**Aqueous two-phase extraction of capsanthin and capsaicin from red pepper.** The phase diagram

of acetone/ $K_2HPO_4$  ATPS was determined by the turbidity titration method (TAN *et al.* 2002). The total weight of water added was measured exactly at different phase transition points, and the concentrations of acetone and  $K_2HPO_4$  at different phase transition points were calculated. The phase diagram curve was plotted (Figure 1).

Acetone is often used for capsanthin extraction (KIM *et al.* 2002; GALLARDO-GUERRERO *et al.* 2010). A molecule of capsanthin contains a phenolic hydroxyl group and an amide bond. Capsaicin is a weak polar acidic compound soluble in alkaline aqueous solutions (SASS *et al.* 1977). Inorganic salts should be distinguished from alkaline salts, such as sodium carbonate, sodium hydroxide, and potassium hydrogen phosphate. Given that dipotassium hydrogen phosphate has a higher solubility at room temperature and is easy to use for phase separation,  $K_2HPO_4$  and acetone were used to establish ATPS.

The influences of acetone and  $K_2HPO_4$  concentrations on the extraction of capsanthin and capsaicin were investigated based on the phase diagram of acetone/ $K_2HPO_4$  ATPS (Figure 1). As shown in Figure 2, the yield of capsanthin increased with the mass fractions of acetone and  $K_2HPO_4$ , and the maximum yield was 44.57% in the ATPS of 30% (w/w) acetone/25% (w/w)  $K_2HPO_4$ . The effect of  $K_2HPO_4$  appeared to be stronger than that of acetone when the mass fractions of acetone were increased from 25% to 30% (w/w). We thought the reason was that the high concentration of salt was conducive to the dissolution of capsanthin. Furthermore, the recovery of capsanthin was 100% in the acetone phase because of the absence of capsanthin in the water phase. Therefore, the higher concentration of acetone was beneficial to the extraction of

capsanthin. When the concentration of  $K_2HPO_4$  was increased, the salt took more water molecules into the bottom phase. As a result, the concentrations of acetone and capsanthin in the top phase increased.

As shown in Figure 3, the yield of capsaicin initially increased and then declined when the mass fractions of acetone increased. A modest increase in the concentration of acetone was conducive to the extraction of capsaicin, so there was a rising trend at first. As the acetone concentration continued to increase, water in the acetone phase was enhanced, the volume of the bottom phase decreased and the concentration of  $K_2HPO_4$  was increased in the water phase. So capsaicin increased in the two-phase interface and declined in the acetone phase when the mass fractions of acetone continued to increase.

However, the yield of capsaicin decreased consistently when the mass fractions of  $K_2HPO_4$  were increased. The maximum yield of capsaicin was 66.15% in the ATPS of 20% (w/w) acetone/15% (w/w)  $K_2HPO_4$ . Conversely, the effect of pH on the solubility of capsaicin is also an important factor (DENG *et al.* 2008). As the concentration of  $K_2HPO_4$  was increased in the water phase, the pH value also increased. Capsaicin dissolved in the alkaline aqueous solution at  $pH > 11$  but existed in the interface of the aqueous phase at  $pH < 11$ . In the described cases, the pH of the water phase in ATPS was less than 11. Therefore, capsaicin increased in the two-phase interface and decreased in the top phase when the concentration of  $K_2HPO_4$  was enhanced.

**Partitioning behaviour of capsanthin and capsaicin in TLPS.** Capsanthin is a fat-soluble pigment that dissolves in *n*-hexane and petroleum ether. However, capsaicin has low solubility in hydrophobic solvents. Three liquid phases could be formed

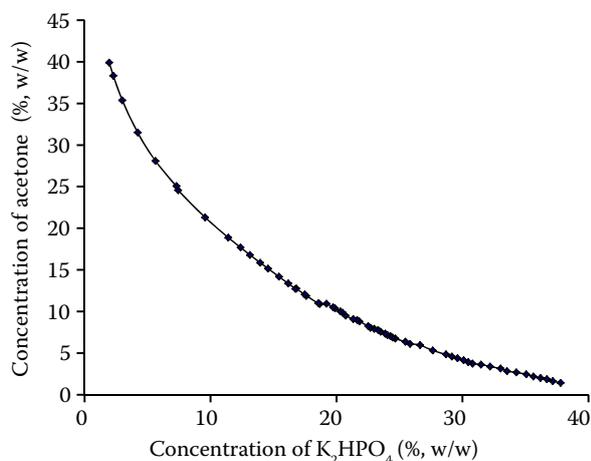


Figure 1. Phase diagram of the acetone/  $K_2HPO_4$  system

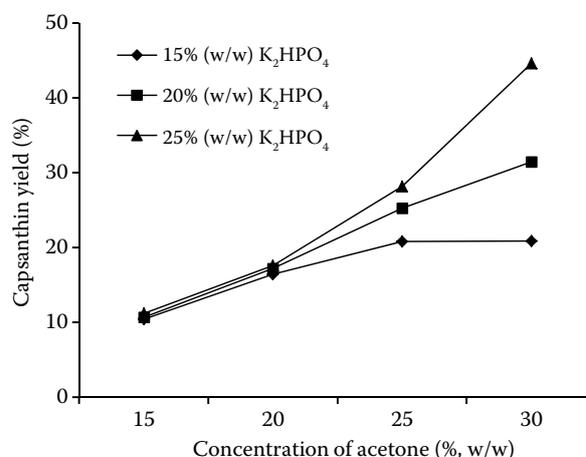


Figure 2. Effect of acetone and  $K_2HPO_4$  concentrations on the extraction of capsanthin in the ATPS

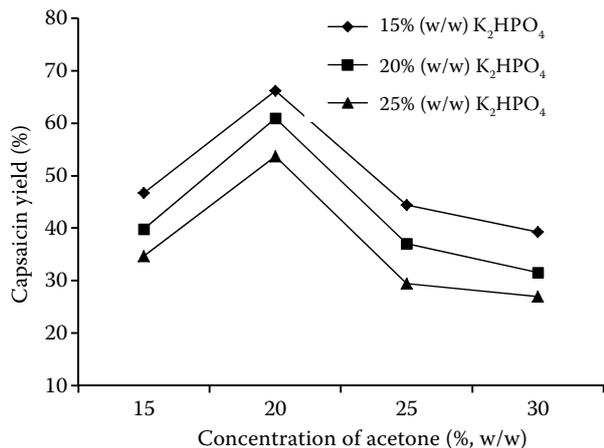


Figure 3. Effect of acetone and K<sub>2</sub>HPO<sub>4</sub> concentrations on the extraction of capsaicin in the ATPS

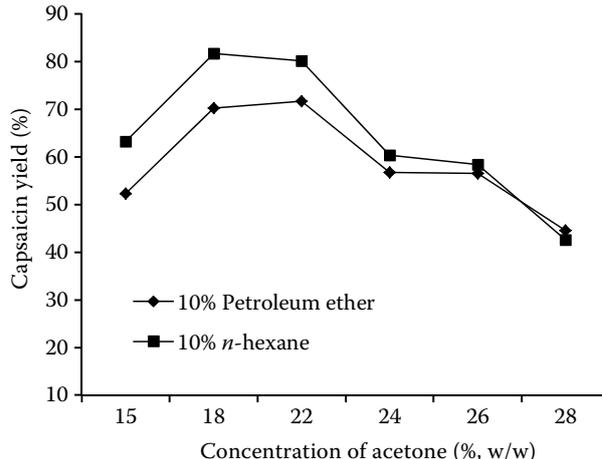


Figure 4. Effect of acetone concentration on the partitioning behaviour of capsaicin in the TLPS

when 10% to 14% (w/w) of *n*-hexane or petroleum ether was added into the ATPS of 15% to 28% (w/w) acetone/20% (w/w) K<sub>2</sub>HPO<sub>4</sub>. According to the phase ratio, a suitable concentration of *n*-hexane or petroleum ether for the separation and enrichment of capsaicin and capsanthin was selected at 10% (w/w). The effect of acetone concentration on the partitioning behaviour of capsanthin and capsaicin was studied in the TLPS of 20% (w/w) K<sub>2</sub>HPO<sub>4</sub>/10% (w/w) *n*-hexane or petroleum ether.

As shown in Figure 4, capsaicin was mainly distributed in the acetone phase, less than 0.01% in the *n*-hexane phase, and undetectable at pH < 11 in the water phase. The capsaicin content in the acetone phase slightly increased and then decreased with increasing acetone concentration. The yield of capsaicin was higher (80.17–81.73%) in an acetone concentration

range of 18% to 22% (w/w). It was also higher in the *n*-hexane TLPS than in the petroleum ether system.

In the TLPS of *n*-hexane/K<sub>2</sub>HPO<sub>4</sub>/acetone, when the concentration of acetone was enhanced, the yield of capsanthin continued to increase, the partition coefficient (*K*) initially increased and then decreased, and the recovery continued to decrease (Figure 5). The effects of acetone concentration in the TLPS of petroleum ether/K<sub>2</sub>HPO<sub>4</sub>/acetone were similar to those in the *n*-hexane/K<sub>2</sub>HPO<sub>4</sub>/acetone system (Figure 6). However, the yield of capsanthin in the latter system (106%) was higher than that in the former system (99%). Considering the yield and *K* of capsanthin, the TLPS of 22% (w/w) acetone/20% (w/w) K<sub>2</sub>HPO<sub>4</sub>/10% (w/w) *n*-hexane was selected for further research.

**Effect of the mass ratio of paprika to TLPS.** The different ratios of paprika to TLPS (1:35, 1:30, 1:25,

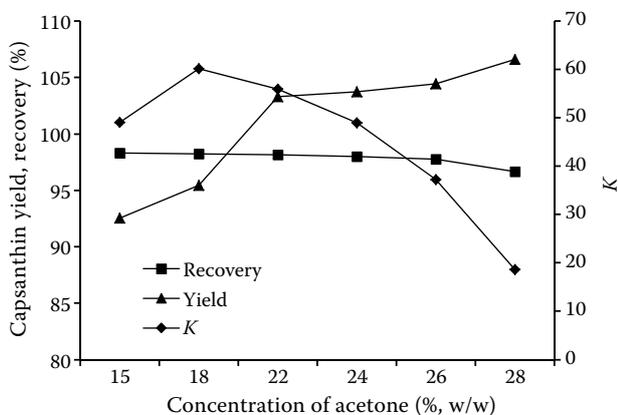


Figure 5. Effect of acetone concentration on the partitioning behaviour of capsanthin in the *n*-hexane/K<sub>2</sub>HPO<sub>4</sub>/acetone system

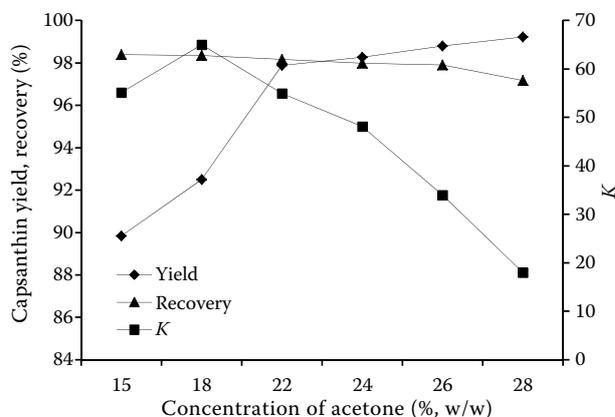


Figure 6. Effect of acetone concentration on the partitioning behaviour of capsanthin in the petroleum ether/K<sub>2</sub>HPO<sub>4</sub>/acetone system

Table 1. Effect of paprika mass ratio on the partitioning behaviour of capsanthin and capsaicin in the TLPS

Ratio	Capsanthin		Capsaicin	
	<i>K</i>	yield (%)	<i>K</i>	yield (%)
1:35	33.10 ± 0.46	98.73 ± 0.61	∞	60.56 ± 1.23
1:30	36.40 ± 0.87	99.11 ± 2.08	∞	75.06 ± 2.26
1:25	42.38 ± 1.15	103.61 ± 2.25	∞	83.24 ± 2.87
1:20	45.67 ± 2.10	103.84 ± 3.17	∞	87.49 ± 3.14
1:16	46.42 ± 2.50	103.92 ± 3.68	∞	88.93 ± 4.05

Values are the mean ± SD from three repetitions

1:20, and 1:16) were investigated (Table 1). The yields of both capsanthin and capsaicin improved when the mass ratio was increased. However, further separation of capsaicin would be disadvantageous if the mass ratio was higher than 1:16 because more paprika and capsanthin would be enriched in the middle phase.

**Effect of extraction procedures on the partitioning behaviour of capsaicin and capsanthin.** As shown in Table 2, the different extraction procedures slightly affected the yield of capsanthin but greatly affected the yield of capsaicin and the *K* of capsanthin. Capsanthin was mainly distributed in the *n*-hexane phase. The partition coefficient of capsanthin in method A and D was lower because when the TLPS was formed, there was more capsanthin in the form of small droplets to be wrapped in the solid phase of paprika, which was disadvantageous to capsanthin enriched in the *n*-hexane phase. However, using method B and C, the ATPS was formed at first. Capsanthin was relatively easy to enter from top phase of ATPS to *n*-hexane phase of TLPS when adding a third solvent to form the TLPS. Capsaicin was mainly distributed in the acetone phase, there was no such situation. The experimental results demonstrated that the lixiviation of capsanthin was faster than

Table 2. Effect of TLPE extraction procedure on the partitioning behaviour of capsanthin and capsaicin in the TLPS

Extraction procedure	Capsanthin		Capsaicin	
	<i>K</i>	yield (%)	<i>K</i>	yield (%)
A	25.95 ± 0.53	104.53 ± 2.10	–	88.52 ± 2.17
B	38.27 ± 1.21	104.82 ± 3.13	–	82.70 ± 2.08
C	34.12 ± 1.16	103.78 ± 2.16	–	49.86 ± 2.45
D	27.13 ± 0.66	102.96 ± 3.11	–	36.33 ± 1.18

Values are the mean ± SD from three repetitions

that of capsaicin. Meanwhile, acetone favoured the lixiviation of capsanthin and capsaicin.

**Comparison of TLPE and conventional solvent extraction.** As shown in Table 3, the yield of capsanthin in TLPE was better than that in the conventional solvent extraction (ZHAO *et al.* 2004). The yield of capsaicin was slightly lower in TLPE than in the traditional extraction method (DENG *et al.* 2008). However, TLPE is still advantageous because it requires a low solvent content at room temperature and a short extraction time. More importantly, TLPE can realise the extraction and separation of capsanthin and capsaicin in a single step.

## CONCLUSIONS

TLPS consisting of *n*-hexane, acetone, K<sub>2</sub>HPO<sub>4</sub>, and water was successfully used to extract capsanthin and capsaicin from red pepper. Capsanthin and capsaicin were partitioned mainly into the *n*-hexane and acetone phases, respectively. They could be easily obtained after removing the solvent for further purification. TLPE was an effective extraction and separation method, especially for natural active ingredients with different polarities in plants.

Table 3. Comparison of different extraction methods

Extraction method	Temperature (°C)	Time (h)	Extract (ml)	Capsanthin			Capsaicin		
				concentration (mg/g)	<i>K</i>	yield (%)	concentration (mg/g)	<i>K</i>	yield (%)
A	30	6	92.5	32.45	–	100 <sup>a</sup>	–	–	–
B	60	4	13.8	–	–	–	14.12	–	100 <sup>b</sup>
C	25	0.5	9 <sup>c</sup> :7.8 <sup>d</sup>	34.09	37.40	105.05	12.37	–	87.61

Data are means from three different experiments; <sup>a</sup>reference set as 100%; <sup>b</sup>reference set as 100%; <sup>c</sup>volume of top phase; <sup>d</sup>volume of middle phase; (A) Extraction with 95% acetone (25 ml/g paprika) at 30°C for 3 h, extraction was conducted twice; (B) Extraction with 95% ethanol (5 ml/g paprika) at 60°C for 2 h, stirring speed was 200 r/min, extraction was conducted twice; (C) Extraction with 22% (w/w) acetone/20% (w/w) K<sub>2</sub>HPO<sub>4</sub>/10% (w/w) *n*-hexane (mass ratio = 1:20) at 25°C for 10 min, extraction was conducted once

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