# Simultaneous micronization and purification of bioactive fraction by supercritical antisolvent technology

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## Abstract

Simultaneous micronization and purification of DLBS3233 bioactive fraction, a combination of two Indonesian herbals Lagerstroemia speciosa and Cinnamomum burmannii has been successfully performed via supercritical anti-solvent (SAS) technology. The objective of the present study was to investigate the effectiveness of SAS technology to micronize and reduce coumarin content of DLBS3233. The effects of four SAS process parameters, i.e. pressure, temperature, concentration and solution flow rate on particle formation were investigated. In SAS process, DLBS3233 was dissolved in dimethylformamide (DMF) as the liquid solvent. The solution was then pumped through a nozzle into a chamber simultaneously with supercritical carbon dioxide (SC-CO2) which acts as the anti-solvent, resulting in DLBS3233 precipitation. Physicochemical properties of unprocessed DLBS3233 and SAS-processed DLBS3233 particles were analyzed using scanning electron microscopy (SEM) and high pressure liquid chromatography (HPLC). Total polyphenol content (TPC) was also analyzed. Particles with mean particle size ranging from $0.107\pm0.028$ $\mu$m to $0.298\pm0.138$ $\mu$m were obtained by varying the process parameters. SAS-processed DLBS3233 particles showed no coumarin content in all experiments studied in this work. Results of TPC analysis revealed no significant change in SAS-processed DLBS3233 particles compared to unprocessed DLBS3233. Nano-sized DLBS3233 particles with no coumarin content have been successfully produced using SAS process. This study demonstrates the ability of SAS for processing herbal medicine in single step process.

**Key words:** DLBS3233, herbal medicine, micronization, purification, supercritical antisolvent

## INTRODUCTION

During the past few years, there has been a growing interest in the use of natural products, especially those derived from plants.[1] Traditional herbal medicine has received significant attention as an alternative therapy to synthetic medicine due to the consideration of possible adverse reactions and also as a substitution when synthetic medicine does not give beneficial effects.[2] The common dosage forms of traditional herbal medicines are tablet and capsule. Due to the rough content of its active component in crude extract, the recommended dose is usually large, and it may cause inconvenience for several patients. Therefore, it is

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considerably important to improve the performance and effectiveness of traditional herbal medicine.

In recent years, the development of herbal medicines with micro- or nano-sized particles has attracted much attention. Various technologies have been used for this purpose, i.e. high-pressure homogenization, supercritical fluid (SCF), and emulsification process.[3,4] Herbal medicines with smaller and more uniform size distribution showed several advantages, including increased solubility, improved dissolution rate in biological environment, lower dosage requirement, less side effects, and improved bioavailability of herbal medicines.[5,6] SCF process is an alternative to conventional methods for processing herbal medicines and removing organic solvent residues.[7] The most commonly used SCF is carbon dioxide ($CO_2$), due to its low critical properties, including critical temperature ($T_c$) and critical pressure ($P_c$), and safety considerations. Among various SCF particle formation techniques, supercritical antisolvent (SAS) process has widely been applied for active pharmaceutical ingredients with limited solubility in supercritical $CO_2$. In this process, organic solvent is pumped simultaneously through a nozzle into the chamber with an SCF, which acts as an antisolvent. The particles are generated due to the supersaturation of solute within the solution droplets.[8] In particular, the application of SCF technology in the precipitation of natural or herbal medicine has been studied. *Manilkara kauki* bioactive fraction (DLBS2347) micronization using SAS has been studied by Widjojokusumo *et al*.,[9] Charoenchaitrakool *et al*., and Chen *et al*. Imsanguan *et al*. studied andrographolide precipitation using SCF as antisolvent.[10-12] Zhao *et al*. studied the SAS precipitation of 10-hydroxycamptothecin, camptothecin, and *Ginkgo biloba* extract.[13-15]

Several herbal medicines based on natural products have been developed in our laboratory, such as DLBS3233, DLBS1442, DLBS1425, and DLBS4847.[16-19] The clinical and preclinical benefits have also been investigated.[16,18,20,21] 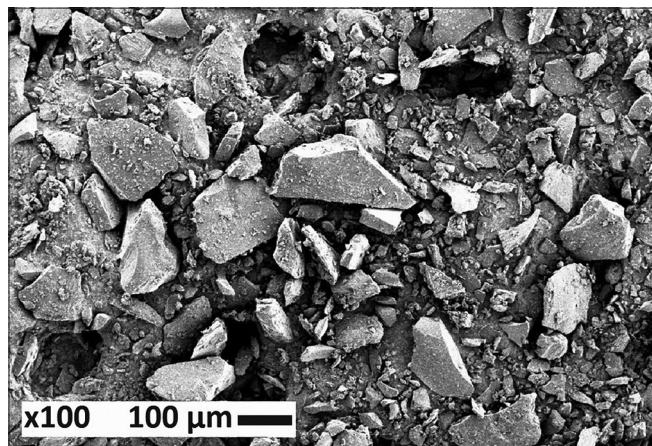DLBS3233 [Figure 1], a herbal combination of *Lagerstroemia speciosa* and *Cinnamomum burmannii* (mass ratio of *L. speciosa* and *C. burmannii* was 1:3 [% w/w]), is a bioactive fraction developed in our laboratory that works as a novel insulin sensitizer.[16,20,21] Due to the coumarin [Figure 2] content in the raw material of *C. burmannii*, preparation of DLBS3233 required multiple steps including percolation, evaporation, drying, and milling process. Coumarin was known to cause liver and kidney damage in rats and mice, and there were isolated incidents of similar hepatotoxicity in human.[22,23] Several studies have been conducted to eliminate or reduce coumarin content from a substance. One of the alternative methods was supercritical fluid extraction (SCFE) technology. Teng *et al*. studied coumarin extraction from the fruit peel of *Citrus maxima* using SCFE.[24] Rodrigues *et al*. also studied SCFE process of coumarin from emburana (*Torresea cearensis*) seeds.[25]

In this study, the effectiveness of SAS technology using $CO_2$ as antisolvent to micronize and reduce coumarin content in DLBS3233 was investigated. The effects of SAS operating condition on the micronization and purification process were studied. Physicochemical properties of processed material were analyzed using scanning electron microscopy (SEM) and high-performance liquid chromatography (HPLC). Comparison of total polyphenol content (TPC) between unprocessed DLBS3233 and SAS-processed DLBS3233 particles was also performed.

## MATERIALS AND METHODS

### Materials
DLBS3233 was obtained from Pharmacognosy and Extraction Laboratory (DLBS, Cikarang, Indonesia). DLBS3233 is brown in color with coumarin content of 0.4 wt%. DLBS3233 was prepared by conventional extraction and drying process. Each dried plant material (at a mass ratio of 1:3 (% w/w) of *L. speciosa* and *C. burmannii*) was alternately mixed and extracted simultaneously in drinking water using percolation technique at 70°C. The resulting micelles were then concentrated using a Rotavapor (Büchi, Switzerland) at 70°C until 40%–50% of total solid was obtained. The concentrated extract was subsequently dried using vacuum oven for 1 night. The dried extract was collected and placed in the glass vial for further analysis. High purity of $CO_2$ (purity of 99.95%) was purchased from PT Intergas (Jakarta, Indonesia). Dimethylformamide (DMF-ACS grade), methanol (HPLC grade), and acetonitrile (HPLC grade) were obtained from J.T. Baker, Inc. (NJ, USA). Polytetrafluoroethylene (PTFE)
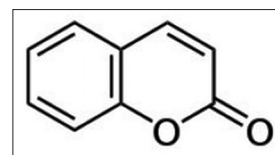


**Figure 1:** Scanning electron microscope images of unprocessed DLBS3233 extract



**Figure 2:** Chemical structure of coumarin

membrane with a size of 0.22 μm was purchased from Fioroni Filters (Ingre, France).

## Methods

### Supercritical antisolvent apparatus

SAS process was conducted using a custom-built SAS apparatus as shown in Figure 3. Solution reservoir (9) was a 110 ml size Pyrex dropping funnel. High-pressure pump for $CO_2$ (3) was a Thar P-50 pump (Thar Technology, PA, USA). High-pressure pump for solution (8) was a Lab Alliance 1200 series (Lab Alliance, PA, USA). The precipitation chamber (7) was made from stainless steel 316 (SS316) with 100 ml internal volume and water jacket to control the temperature of precipitator. A pair of glass windows was installed in front and back side of precipitation chamber to observe the process of particle formation. A stainless steel capillary tube with internal diameter of $2.54 \times 10^{-4}$ m was used as a nozzle, and it was placed on the top of precipitation chamber. The $CO_2$ precooler (2) and preheater (6) were shell and tube types, respectively. The inner tube was a coiled tube with 0.6 m in length and $3.175 \times 10^{-3}$ m in outside diameter that made from SS316. The shell was made from SS316 with 0.11 m in diameter and 0.25 m in length. The products were filtered using 0.22 μm PTFE membrane filter (10). The pressure of the precipitation chamber was controlled using a model 26-1721-24 back-pressure regulator (11) manufactured by Tescom, Co. (MN, USA). The precooler and $CO_2$ pump temperature were maintained using cooling circulator (4).

### Supercritical antisolvent process

The solution of DLBS3233 in DMF was prepared at a concentration of 20 mg/ml. The SAS process was initiated
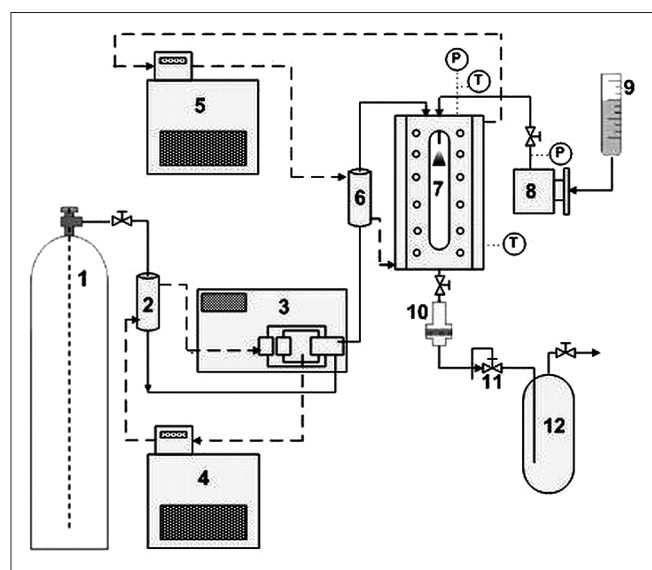


**Figure 3:** Schematic diagram of custom-built supercritical antisolvent apparatus (1) $CO_2$ cylinder, (2) precooler, (3) high-pressure pump for carbon dioxide, (4) cooling bath, (5) heating bath, (6) preheater, (7) precipitation chamber, (8) high-pressure pump for solution, (9) solution reservoir, (10) filter, (11) back-pressure regulator, and (12) separator

by supplying fresh $CO_2$ into precipitation chamber. Once the temperature reached steady state, the pressure was tuned by the regulator to experimental condition while $CO_2$ was supplied continuously using a high-pressure pump. DLBS3233 solution in DMF was then sprayed into the precipitator at a desired flow rate. Precipitated fine particles were collected in the filter. After all solutions were sprayed, $CO_2$ was supplied continuously for 60 min to remove all remaining solvent in the product inside the precipitation chamber and filter. Products were collected from the filter after depressurizing the precipitation chamber.

### Scanning electron microscope analysis

Morphology of the samples was analyzed using a JEOL JSM-6510 scanning electron microscope (JEOL Ltd., Tokyo, Japan). Samples were mounted on a double-faced adhesive tape sputtered with platinum. Scanning electron photographs were taken at an accelerating voltage of 5 kV. The particle size (PS) and PS distribution were determined by microscopic quantitative analysis of at least 150 particles in the SEM image, and these values were used to obtain the corresponding statistics.

### High-performance liquid chromatography analysis

Coumarin content in unprocessed DLBS3233 and SAS-processed DLBS3233 particles was determined by a Waters Alliance HPLC system which includes a Waters e2695 separation module, a Waters 2489 UV detector, and a 4.6 mm × 150 mm dC18 Atlantis, 5 μm column (Waters Corporation, Milford, MA, USA). The mobile phase consisted of water and acetonitrile (60/40, %v/v) with a flow rate of 1.0 ml/min. In each analysis, 20 μl of DLBS3233 solution in methanol was injected, and coumarin was detected at 274 nm. Data acquisition and analysis were performed using Empower 2.0 software (Waters Corporation, Milford, MA, USA).

### Total polyphenol content analysis

Total amount of phenolic in the extracts was determined using Folin–Ciocalteu reagent. Gallic acid was used as a standard. Briefly, 1 ml of the diluted sample was transferred to 25 ml volumetric flask containing 9 ml $CO_2$-free deionized (DI) water and 1.0 ml Folin–Ciocalteu reagent. After 5 min, 10 ml sodium carbonate solution (7% w/v) was added to the sample, and the total volume was made up to 25 ml by adding $CO_2$-free DI water. The volumetric flask was then allowed to stand at room temperature in dark conditions for 2 h before absorbance measurement at 750 nm. The concentrations of TPC were quantified using gallic acid calibration curve.

## RESULTS AND DISCUSSION

### Effect of processing parameters in supercritical antisolvent process of DLBS3233

In SAS process, the size and morphology of the particles can be manipulated by adjusting the process parameters,

including pressure, temperature, $CO_2$ flow rate, solution flow rate, and drug concentration. For this reason, in this study, the influence of different SAS process parameters on the PS of DLBS3233 was studied, including pressure ($P$) between 100 and 200 bar, temperature ($T$) between 35°C and 55°C, solution flow rate ($Q_L$) between 0.53 and 2.19 ml/min, and DLBS3233 concentration in DMF ($C$) between 5 and 20 mg/ml. The $CO_2$ flow rate ($Q_{CO_2}$) and washing time were kept constant at 30 g/min and 60 min, respectively. The experimental conditions for SAS experiments are summarized in Table 1. All the SAS experiments in this work led to the successful precipitation of DLBS3233, as verified by the results in Table 1 and Figure 4. It can be seen that the morphology of unprocessed and SAS-processed DLBS3233 particles was significantly different. From Figure 1, unprocessed DLBS3233 particles were irregular and ranging in size between 10 and 200 μm. The SEM images of SAS-processed DLBS3233 particles showed the formation of nearly spherical, nano-sized particles with mean PS of 0.107–0.298 μm [Figure 4]. In general, mean PS was reduced by approximately 400 times regarding to the raw material.

The effect of pressure on PS is seen by comparing runs 1–3 [Table 1]. As shown in Table 1 and Figure 4 (runs 1–3), an increase in pressure led to a decrease in PS. As the density of $CO_2$ is increased at high-pressure, large deforming forces will further break the existing droplets into smaller size. In addition, as $CO_2$ has a better solubility in the liquid solvent with increased pressure, the increasing quantities of supercritical carbon dioxide (SC-$CO_2$) were rapidly solubilized in the solvent, lowering the solvent power toward the solute and substantially caused higher degree of supersaturation of the expanded liquid solution, and resulting in the precipitation of smaller particles.[26,27]

**Table 1: Operation condition of DLBS3233 precipitation using supercritical antisolvent**

| Run | Pressure (bar) | Temperature (°C) | $Q_{CO_2}$ (g/min) | $Q_L$ (ml/min) | C (mg/ml) | PS (μm) |
|---|---|---|---|---|---|---|
| 1 | 100 | 35 | 30 | 1.07 | 20 | 0.170±0.055 |
| 2 | 150 | 35 | 30 | 1.07 | 20 | 0.122±0.035 |
| 3 | 200 | 35 | 30 | 1.07 | 20 | 0.107±0.028 |
| 4 | 150 | 45 | 30 | 1.07 | 20 | 0.161±0.061 |
| 5 | 150 | 55 | 30 | 1.07 | 20 | 0.189±0.062 |
| 6 | 150 | 35 | 30 | 0.53 | 20 | 0.130±0.035 |
| 7 | 150 | 35 | 30 | 2.19 | 20 | 0.129±0.054 |
| 8 | 150 | 35 | 30 | 1.07 | 5 | 0.298±0.138 |
| 9 | 150 | 35 | 30 | 1.07 | 10 | 0.238±0.120 |

$Q_{CO_2}$: $CO_2$ flow rate, $Q_L$: Solution flow rate, *C*: DLBS3233 concentration in DMF, PS: Particle size, DMF: Dimethylformamide
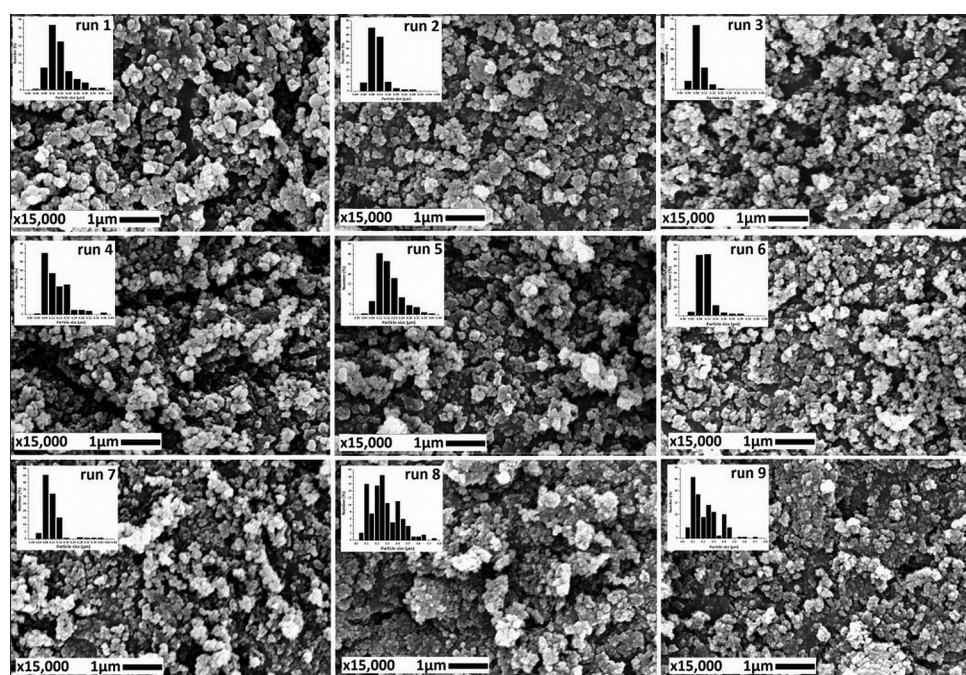


**Figure 4:** Scanning electron microscope images of supercritical antisolvent-processed DLBS3233 particles (curves of particle size distribution are shown in the corner)

To investigate the effect of operating temperature on precipitated particles, three experiments were conducted at temperatures of 35°C–55°C while constantly maintaining all other variables as shown in runs 2, 4, and 5 [Table 1]. The observation concluded that increased temperature led to an increase in PS. The solubility of DLBS3233 in liquid solvent was increased at high temperature, thus decreasing the degree of supersaturation. Consequently, the PS of the processed DLBS3233 was increased due to reduced formation of the number of nuclei.[28]

Experiment runs 2, 6, and 7 were performed at $Q_L$ of 0.53–2.19 ml/min to explore the effect of solution flow rate. Based on the results on Table 1 and Figure 4, it can be clearly seen that solution flow rate did not significantly affect PS. In SAS process, increased solution flow rate usually showed competing effects. Higher flow rates decreased the mass transfer rates of organic solvent out of the droplets, thus reduced the supersaturation ratio and resulted in larger particles. Meanwhile, as the solution feed rate increased, the Weber number was also increased. Higher Weber number resulted in smaller liquid droplets of the solution, followed by the formation of small PS.[29]

DLBS3233 was also precipitated at various concentrations of 5–20 mg/ml to investigate the effect of solution concentration on PS of DLBS3233 after SAS process. It is shown in Table 1 and Figure 4 that an increase in solution concentration led to smaller PS with narrower distribution. For higher concentrations, an intense nucleation was occurred with predominance in particle growth and smaller particles with uniform size.[30]

## Coumarin content analysis of supercritical antisolvent-processed DLBS3233 particles

The results of HPLC analysis showed that there were significant reductions of coumarin content in SAS-processed DLBS3233. The coumarin content in unprocessed DLBS3233 was 0.4 wt% whereas SAS-processed DLBS3233 particles showed no coumarin content in all experiments [Figure 5]. These findings indicated that coumarin was soluble in SC-CO$_2$ at all conditions (pressure and temperature) studied in this work.

## Total polyphenol content analysis

The data reported in Table 2 show the comparison of TPC between unprocessed DLBS3233 and SAS-processed
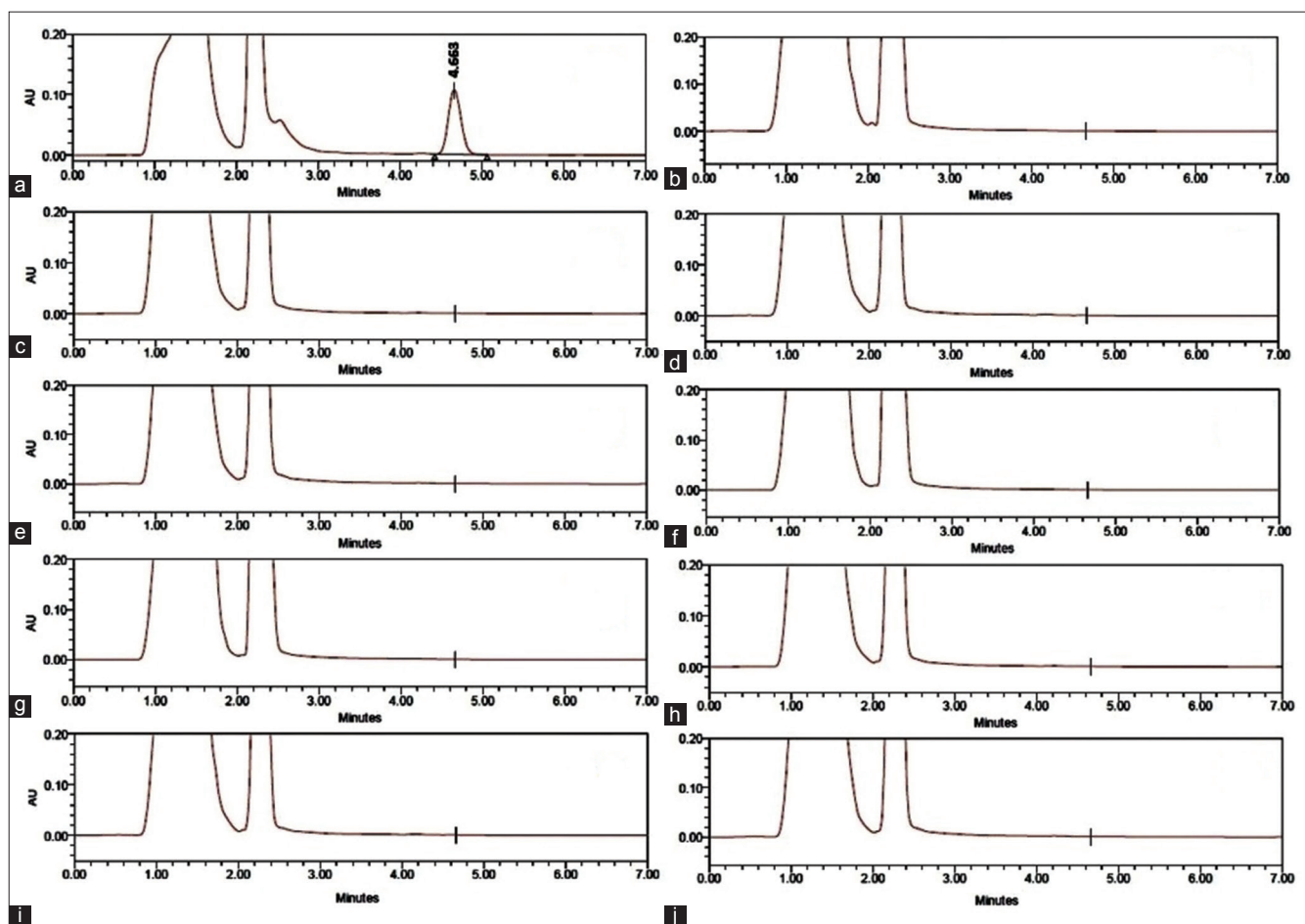


**Figure 5:** High-performance liquid chromatography chromatogram of coumarin content on unprocessed DLBS3233 (a) and supercritical antisolvent-processed DLBS3233 particles run 1 (b), run 2 (c), run 3 (d), run 4 (e), run 5 (f), run 6 (g), run 7 (h), run 8 (i), run 9 (j)

**Table 2: Total polyphenol content of unprocessed DLBS3233 and supercritical antisolvent-processed DLBS3233 particles**

| Sample | Total polyphenol (%) |
| --- | --- |
| DLBS3233 | 34.611 |
| Run 1 | 38.544 |
| Run 2 | 38.937 |
| Run 3 | 38.027 |
| Run 4 | 38.567 |
| Run 5 | 39.611 |
| Run 6 | 35.049 |
| Run 7 | 37.550 |
| Run 8 | 39.505 |
| Run 9 | 38.314 |

Run 1-9: SAS-processed DLBS3233 particles at various operating conditions. SAS: Supercritical antisolvent

DLBS3233 particles at various operating conditions as shown in Table 1. The results showed that there was no significant change in the TPC of DLBS3233 after SAS process.

## CONCLUSION

DLBS3233 has been successfully precipitated using SAS technology. The results showed that the size of precipitated particles was affected by processing conditions. Nano-sized DLBS3233 particles with no coumarin content have been successfully produced using SAS process. This study showed the application of SAS technology for processing herbal medicine in a single-step process.

### Conflicts of interest

There are no conflicts of interest.

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