Central composite design based statistical modelling of curcuminoids extraction from *Curcuma zedoaria* using choline chloride based Natural Deep Eutectic Solvent (NADES)

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**Abstract.** Ionic type of Natural Deep Eutectic Solvents (NADES) consisted of choline chloride and organic acids, i.e., choline chloride-malic acid (CCMA) and choline chloride-citric acid (CCCA), known as the most suitable liquefied solvent for curcuminoids extraction from *Curcuma zedoaria*. A laboratory-scale extraction of curcuminoids from *C. zedoaria* successfully conducted; indicated that solvent per feed ratio (S/F) and extraction time are the two important variables for yielding a high extraction rate of curcuminoids. Therefore, a central composite based design is used to optimize the extraction parameters such as S/F (3/10, 5/10, 7/10), time extraction (18, 20, and 22 h), and stirring speed (200, 250, and 300 rpm) at 500 mL capacity. Stirring speed is the most influential parameter among the studied parameters. However, due to the low bulk density of *C. zedoaria* powder, a homogenous mixture of *C. zedoaria* in the extraction tank was barely achieved; hence a low yield of curcuminoids is obtained.

1. **Introduction**

The World Health Organization (WHO) and the International Cancer Control Union (UICC) predict that in 2030, there will be an increase in cancer patients by 300% worldwide. Almost 20% of them will be in developing countries such as Indonesia. Thus, The Ministry of Health of the Republic of Indonesia declares that cancer is one of the main causes of death.

Curcumin, a kind of curcuminoids, is commonly found in the Zingiberaceae family such *Curcuma zedoaria*. It is known as an anticancer agent [1], antioxidant, etc. However, its efficacy and safety are good, curcumin currently not yet approved as an anticancer therapeutic agent. The limited supply of curcumin extract for medicinal purposes is one of the reasons [2]. Since human blood contains approximately 90% of water [3], low solubility of curcumin in the water (pH = 7.3), 4 ppb (4 µg / L), also a constraint factor. Therefore, the carrier material of curcumin that can maximally release curcumin needs to be studied to facilitate the oral delivery of curcumin in the human body maximally.

Various extraction processes of curcumin have been conducted for obtaining a high yield of curcumin. Organic solvents (such as ethanol, methanol, ethanol-water, etc.) are commonly used as solvents. However, organic solvents leave residues in the crude extract. They potentially deposited in the body. Therefore, an environmentally friendly solvent, which is health and save, is necessary to use in the extraction process especially in pharmaceutical industries.

The next generation of ionic liquids (IL), called eutectic solvents (DES), consisted of low toxicity chemical compounds in the form of cations and anions [4]. The main advantages of DES are easy to
make. Just by melting two chemical compounds at high purity with moderate heat. The suitability of DES for biological applications can be seen at dissolving enzymes into DES [5]. There is no change in the enzyme conformation. The enzyme activity can be controlled by water addition. Enzyme deactivation can be made by minimizing the water content by means freeze-drying the enzyme and reactivating it back by diluting with water [5]. When DES consisted of primary metabolites such as sugar, sugar alcohol, polyalcohol, organic bases, organic acids, and amino acids, they referred to as Natural Deep Eutectic Solvent (NADES) [6]. Various macro- and micro-molecules are well-dissolved NADES such as gluten and DNA [7]. A micro-molecules such routine, quercetin, cinnamic acid, taxol, carthamin, ginkgolide B provide good solubility in some types of NADES [7]. Some of them showed a higher solubility in NADES compare in water and methanol. NADES proline-malic acid (PMH) and lactic-glucose (LGH) are both used to extract phenolic, cartormin, and carthamin from safflower (Flos Carthami) and corolla from Carthamus tinctorius L. [8]. These safflower natural dyes showed high solubility in PMH and LGH. Sugar-based NADES reported to be stable to store natural phenolic pigments, cyanidin, both at higher temperatures and at longer storage times [8]. Moreover, Zullaikah et al. [9] showed suitability of NADES for extracting curcuminoids. Neutral type of NADES (FS-H2O), ionic type of NADES (CCMA-H2O) and acidic type of NADES (CAS-H2O and MAS-H2O) are better-extracted curcuminoids from Curcuma mangga compared to other kinds of NADES. The highest yield of curcuminoids given by CCMA-H2O (1: 1: 2) of 0.355 mg curcuminoids/g dry powder of C. mangga.

The extraction process with a high yield of curcuminoids requires optimum conditions of the extraction parameters such as particles size, type of solvents/NADES, porosity and diffusivity, pH, extraction time, agitation speed, temperature, solvent to feed (S/F) ratio, and mode of the extraction process. Therefore, to increase the extractability of curcuminoids from Curcuma powder by NADES the optimum condition of the extraction parameters need to be studied. This study focused on optimizing the extraction parameters of Curcuma zedoaria, i.e., extraction time, agitation speed, and solvent to feed (S/F) ratio, using Response Surface Methodology (RSM).

2. Materials and Methodology

2.1. Reagents and chemicals
Curcuminoids standard purchased from Merck (Darmstadt, Germany) as well as solvents such as acetonitrile, acetic acid (HOAc), and methanol (MeOH). All the solvents are in High Pure Analytical grade. While an individual compound of NADES such as choline chloride and malic acid purchased from Sigma Aldrich (St. Louis, MO, USA). While citric acid in food-grade purchased at Surabaya local market (Gajah, Jakarta, Indonesia). Buffer solution (pH = 2.0) from Mediss (India). Moreover, fine powder of Curcuma zedoaria kindly donated from Sari Herbal (Sukun, Malang, Indonesia).

2.2. Natural Deep Eutectic Solvents (NADES) preparation
All NADES compounds used in this study were prepared based on the method by Rachmaniah et al. [10], which modification method is described earlier [7].

2.3. Extraction of curcuminoids using Natural Deep Eutectic Solvents (NADES)
Curcuma zedoaria powder was accurately weighed based on the S/F variable as determined. It was firstly placed in the extractor (amber glass extractor), equipped with a thermometer that set at 40°C. Subsequently, NADES, either CCMA-H2O (1: 1: 18) or CCCA-H2O (1: 1: 18), was added. Each experiment conducted using 200 mL of NADES as a solvent. The extraction was conducted with IKA RW-20 digital rotor (IKA, Staufen, Germany) included its 4-blades propeller IKA R-1342 (IKA, Staufen, Germany). After the extraction is complete, 1 mL sampled and stored at freeze for further step of analysis.
2.4. Analysis of curcuminoids using spectrophotometer

Extracted curcumin was determined by spectrophotometric at $\lambda_{\text{max}} = 425$ nm. As the spectrophotometric method cannot differentiate curcumin, desmethoxycurcumin, and bisdemethoxycurcumin, hence it expressed as total curcuminoids. A 400 µL was accurately pipetted and diluted by adding a mixture of methanol-3% v of HOAc to 2 mL, pH adjusted to pH = 1.8-2.0. Subsequently, the diluted sample was analyzed using a UV-Vis Spectrophotometer T-60 (PG Instruments, Leicestershire, UK). The extracted yield of curcuminoids expressed as a weight of extracted curcuminoids (mg) per dry weight of Curcuma zedoaria powder (g).

2.5. Data analysis using Response Surface Methodology (RSM)

The optimum condition for extraction time, S/F ratio and agitation speed were simulated by responses surface methodology (RSM) with Minitab program.

3. Result and Discussion

### Table 1. Central Composite Matrix Design

| Run Order | Standard Order | Optimized variables | Yield (mg curcuminoids/g) |
|-----------|---------------|---------------------|---------------------------|
| Agitation Speed (rpm) | S/F (mL/mg) | Time (hour) | CCCA-H$_2$O (1:1:18) | CCMA-H$_2$O (1:1:18) |
| 1 | 17 | 250 (0) | 5/10 (0) | 20 (0) | 6.086 | 7.246 |
| 2 | 15 | 250 (0) | 5/10 (0) | 20 (0) | 6.086 | 7.206 |
| 3 | 13 | 250 (0) | 1/6 (-1.68) | 20 (0) | 2.004 | 1.820 |
| 4 | 12 | 250 (0) | 5/10 (0) | 23.36 (+1.68) | 5.069 | 7.066 |
| 5 | 9 | 200 (-1.68) | 5/10 (0) | 20 (0) | 1.534 | 3.829 |
| 6 | 10 | 300 (+1.68) | 5/10 (0) | 20 (0) | 1.534 | 3.469 |
| 7 | 18 | 250 (0) | 5/10 (0) | 20 (0) | 6.086 | 7.266 |
| 8 | 3 | 220 (-1) | 3/10 (-1) | 22 (+1) | 2.653 | 2.645 |
| 9 | 5 | 220 (-1) | 7/10 (+1) | 18 (-1) | 5.738 | 7.039 |
| 10 | 20 | 250 (0) | 5/10 (0) | 20 (0) | 6.086 | 7.226 |
| 11 | 14 | 250 (0) | 5/6 (+1.68) | 20 (0) | 5.424 | 6.168 |
| 12 | 8 | 280 (+1) | 7/10 (+1) | 22 (+1) | 5.835 | 6.787 |
| 13 | 4 | 280 (+1) | 3/10 (-1) | 22 (+1) | 3.818 | 2.597 |
| 14 | 1 | 220 (-1) | 3/10 (-1) | 18 (-1) | 2.501 | 2.609 |
| 15 | 16 | 250 (0) | 5/10 (0) | 20 (0) | 6.086 | 7.326 |
| 16 | 11 | 250 (0) | 5/10 (0) | 16.64 (-1.68) | 4.653 | 3.489 |
| 17 | 7 | 220 (-1) | 7/10 (0) | 22 (+1) | 5.997 | 7.123 |
| 18 | 19 | 250 (0) | 5/10 (0) | 20 (0) | 6.086 | 7.246 |
| 19 | 6 | 280 (+1) | 7/10 (+1) | 18 (-1) | 5.771 | 4.969 |
| 20 | 2 | 280 (+1) | 3/10 (-1) | 18 (-1) | 4.012 | 2.681 |

* not randomized order; * random order; * code on experimental design: -1 low level; 0 mid level, +1 high level

Three variables, i.e., extraction time (h), solvent to feed ratio (S/F), and agitation speed, optimized in these experiments. There are two methods, i.e., Box-Behnken or Central Composite Design, for optimizing two or more variables. However, using Box-Behnken (BB), 15 experiments are required. Whereas 20 experiments are needed when using Central Composite Design (CCD). The CCD method designed 5 levels (-1.68, -1, 0, +1, +1.68) while only 3 levels (-1, 0, +1) for BB. The two additional levels (-1.68 and +1.68) are the extreme conditions (far from the central level, 0). CCD
well applied in continuous experiments such as this study, in which the previous extraction conditions (extraction time, solvent ratio to feed (S/F), and agitation speed) applied as the mid-level (0). The matrix design of the experiments both for CCMA-H$_2$O (1: 1: 18) and CCCA-H$_2$O (1: 1: 18) is displayed in Table 1 as well as yield curcuminoids.

_Curcuma zedoaria_ contains 1.96% of curcuminoids [9]. Applying S/F ratio of 5/10 with 200 mL of NADES as solvent, 400 mg of _C. zedoaria_ powder will be used. Those, maximally 7.840 mg of curcuminoids, will be extracted, by assuming all the curcuminoids extracted. NADES CCMA-H$_2$O (1: 1: 18) successfully recovers 14-37% of curcuminoids from _C. zeodaria_ while CCCA-H$_2$O (1: 1: 18) only 8-31% (Table 1). Even though the recovery is less than 50%; the obtained yield was higher compared to Zullaikah et al. [9]. It only yielded 0.355 mg curcuminoids/g with 4 hours longer of the extraction time (S/F = 3 mL/20 mg, 40 °C, 24 h of extraction). The obtained yield is comparable with microwave-assisted extraction (MAE) of _C. longa_ with ethanol yielded 3.03 mg/g (S/F = 60 mL/0.3 g, 800 W, 2450 MHz, 7 min) [11]. Pulsed ultrasonic-assisted extraction (PUAE) of _C. longa_ with ethanol yielded 10.30 mg/g (S/F = 20 mL/0.1 g, 3/1 s/s pulsed duration/interval time) [11]. Since _C. longa_ contains higher curcuminoids, 4.4% [9], hence only 7% and 23% of the recovery, respectively for MAE and PUAE. In the case of extraction, instead of yield, the selectivity and type of solvent, as well as duration of extraction, were also necessary to be considered to obtain the optimum extraction condition. Mostly, the conventional extraction process i.e. Soxhlet extraction with volatile organic solvents (VOS) such acetone, ethyl acetate, ethanol, methanol, gives a high yield of curcuminoids [12]. 24 mg curcuminoids/g of _C. longa_ powder. However, this type of extraction method is claimed not to be classified as a green process due to the utilization of VOS, time-consuming, usage of huge amounts of hazardous solvent as well as the toxicity of VOS to humans and environment [13].

### Table 2. ANOVA Analysis of Central Composite Design

| Parameters | NADES CCCA-H$_2$O | p-value | NADES CCMA-H$_2$O | p-value |
|------------|-------------------|---------|-------------------|---------|
| Linear     | 0.002             | Linear  | 0.000             |
| Speed      | 0.406             | Speed   | 0.268             |
| Time       | 0.745             | Time    | 0.011             |
| S/F        | 0.000             | S/F     | 0.000             |
| Square     | 0.001             | Square  | 0.000             |
| Speed*Speed| 0.000             | Speed*Speed | 0.000 |
| Time*Time  | 0.487             | Time*Time | 0.007 |
| S/F*S/F    | 0.024             | S/F*S/F | 0.000             |
| 2-Way Interaction | 0.661 | 2-Way Interaction | 0.401 |
| Speed*Time | 0.815             | Speed*Time | 0.427 |
| Speed*S/F  | 0.241             | Speed*S/F | 0.241 |
| Time*S/F   | 0.874             | Time*S/F | 0.341             |

Quadratic polynomial regression model from ANOVA analysis (Table 2) shown significant p-values, both for CCCA-H$_2$O and CCMA-H$_2$O. Those had lower values than the Confidence Interval (CI), ca. 5% significances. Furthermore, the equations for the optimum condition are the following, where A is agitation speed (rpm), B is extraction time (h), and C is S/F ratio (mL/mg).

NADES CCCA-H$_2$O (1:1:18):

$Yield (mg/mg) = \text{-}121.8 + 0.795*\text{A} + 1.77*\text{B} + 32.1*\text{C} - 0.001475*\text{A}^2 - 0.0378*\text{B}^2 - 13.92*\text{C}^2 - 0.00113*\text{A}\times\text{B} - 0.0584*\text{A}\times\text{C} + 0.114*\text{B}\times\text{C}$

(1)
NADES CCMA-H₂O (1:1:18):
Yield (mg curcuminoids/g C. zedoaria) = -133.9 + 0.608*A + 5.22*B + 35.3*C - 0.001315*A² - 0.1520*B² - 26.55*C² + 0.00336*A*B - 0.0506*A*C + 0.609*B*C (2)

Figure 1 is the response surface contour for studied variables versus the yield of curcuminoids (mg/g). Figure 1B approximately representing the ideal surface contour, a capsized bowl-shaped. Both the agitation speed and S/F ratio both have p-values <0.05 in the square parameter (Table 2), indicating that both parameters significantly affect the yield in the quadratic model. In other words, a slight change of both variables will result in a significant change in the yield of curcuminoids. Moreover, the p-value (Table 2) also supported this fact. Fernandez et al. [14] also observed that S/F ratio and Reynold numbers (related to agitation speed) were highly affected by the extraction process in the pilot-scale extraction of Mango leaves. In addition, the agitation speed significantly affects the yield due to the observed vortex formed when extraction conducted at >250 rpm, though at 250 rpm it is still in the transition flow range.

Figure 1. Response surface contour of the yield of curcuminoids (mg/g) with CCMA-H₂O as a NADES solvent: (A) agitation speed (rpm) vs extraction time (hours); (B) agitation speed (rpm) vs S/F ratio (mL/mg); and (C) extraction time (hours) vs S/F ratio (mL/mg).

Meanwhile, Figure 1A and 1C show that the extraction time is less significant to the yield. A doldrums surface contour was reflecting this relation. Extraction time is slightly affecting the yield.
Subsequently, the surface plot looks like a saddle shape. Hence, the S/F ratio variable is the most influential, both in the linear and quadratic models.

3.1. Effect of the extraction time on the yield of curcuminoids
The designed variables of the extraction time are 18 h (-1), 20 h (0), and 22 h (+1) both for CCMA-H$_2$O (1: 1: 18) and CCCA-H$_2$O (1: 1:18). The center level of the extraction time, ca. 20 hours, was selected due to it was yielding the highest yield of curcuminoids at our previous study (data not shown). Longer extraction time will increase the obtained yield. It is possibly due to the longer contacting time between the raw material of C. zedoaria powder and NADES solvent. Hence, the mass transfer of curcuminoids from the materials to the bulk of the liquid solvent will go maximally until reaching a saturation condition of the solvent [14]. Meaning, no mass transfer of curcuminoids from the materials to the bulk of liquid solvent after the saturation condition reached. Since the driving force of curcuminoids concentration approximately zero, the saturation condition observed happens beyond 20 hours of the extraction time. Those, the yield of curcuminoids remain constant and tend to decrease.

3.2. Effect of solvent/feed (S/F) ratio on the yield of curcuminoids
The S/F ratio = 5/10 (5 mL NADES/10 mg Curcuma powder) was chosen. Taking constant a volume of NADES ca. 200 mL, an excessive amount of powder (smaller value of S/F) decreased the contacted surface of powder with the NADES. By having a higher S/F ratio, a lower amount of powder applied. Higher S/F value may give a higher concentration gradient between the curcuminoids inside and at the surface of the solid (powder), which may result in a higher extraction rate and finally increase the obtained yield of curcuminoids. Hence, a lower yield value of curcuminoids resulted and vice versa. The resulted optimum condition of S/F is 7/10.

3.3. Effect of agitation speed on the yield of curcuminoids
The agitation conducted by applying a 4-blades propeller type mixer. It was chosen since it is suitable for the viscosity of NADES and produces an axial agitation pattern for homogenizing the C. zedoaria powder throughout the solvent [15]. The agitation speed significantly affects the obtained yield. A significant correlation between an agitation speed and the yield of curcuminoids shown in the ANOVA analysis (Table 2) for both types of NADES. The p-value of the agitation speed as a quadratic correlation is lower values than CI ca. 5% significances.

A significant increase in the yield of curcuminoids obtained at 200-250 rpm though a dispersed of C. zedoaria powder observed when agitated at 200-250 rpm. However, a vortex is present when agitated >250 rpm, resulting in a poor homogenization [15]. Thus, lower yield of curcuminoids is obtained.

3.4. Optimum conditions of the extraction
The optimum conditions of the extraction optimized using Minitab. The optimum value of time extraction, S/F ratio, and agitation speed shown in Table 3. Both types of NADES, more or less, have similar optimum conditions. However, CCMA-H$_2$O yielded higher curcuminoids. It is possibly due to the different characteristics of both NADES, such as density, viscosity, and acidity [7, 16].

Both CCCA-H$_2$O (1: 1: 18) and CCMA-H$_2$O (1: 1: 18), have lower viscosity compared to sugar-based on NADES [4]. A lower viscosity solvent with a higher diffusion coefficient increases the mass transfer, enhances the solubility and finally the extraction rate of curcuminoids [16]. Moreover, a high molar ratio of water, ca. 18 molar ratio, resulting in a density approximately to water. They are 1.1164 and 1.1431 g/mL, respectively, for CCCA-H$_2$O (1: 1: 18) and CCMA-H$_2$O (1: 1: 18). NADES CCCA-H$_2$O (1: 1: 18) and CCMA-H$_2$O (1: 1: 18) have viscosity 8,914 cP and 10,885 cP, respectively, resulting in a higher yield of curcuminoids in CCMA-H$_2$O than CCCA-H$_2$O (1: 1: 18).
In the case of polarity, both of the selected NADES have more or less a similar polarity, 47.8 kcal/mol and 47.91 kcal/mol (polarity is expressed as $E_{NR}$), respectively for CCMA-$H_2O$ (1:1:18) and CCCA-$H_2O$ (1:1:18). Therefore, the polarity does not majorly affect the curcuminoids yield.

| NADES      | Agitation Speed (rpm) | S/F (mL/mg) | Time (h) | Predicted yield of curcuminoids (mg/g) | $R^2$  |
|------------|-----------------------|-------------|----------|---------------------------------------|--------|
| CCCA-$H_2O$ | 247.45                | 0.721       | 20.85    | 6.701                                 | 0.885  |
| CCMA-$H_2O$ | 245.41                | 0.673       | 21.26    | 8.163                                 | 0.944  |

Based on the optimal variables obtained from the Minitab application (Table 3), verification experiments carried out in triplicates. Respectively, 12.953±0.135 and 15.239±0.121 mg/g were obtained for CCCA-$H_2O$ (1:1:18) and CCMA-$H_2O$ (1:1:18). Unfortunately, the obtained yield of curcuminoids at the optimum conditions was significantly different from the models. CCD models of RSM are well-optimized the condition of the curcuminoids extraction from C. zedoaria. However, the extraction process itself was not well-modeled by the CCD.

4. Conclusion

Extraction time (h), solvent to feed (S/F) ratio, and agitation speed (rpm) of curcuminoids extraction from Curcuma zedoaria optimized by means central composite design (CCD) with response surface methodology. The optimum condition for CCCA-$H_2O$ (1:1:18) were S/F = 0.721 (mL/mg), 20.85 h extraction time and 247.45 rpm. While S/F = 0.673 (mL/mg), 21.26 h extraction time and 245.41 rpm for CCMA-$H_2O$ (1:1:18). However, the verification experiments at optimum condition yielded 12.953±0.135 and 15.239±0.121 mg/g, respectively, for CCCA-$H_2O$ (1:1:18) and CCMA-$H_2O$ (1:1:18), of curcuminoids. CCD models of RSM can well optimize the condition of the curcuminoids extraction from C. zedoaria ($R^2 = 0.885$-$0.944$). Meanwhile, it does not well model the extraction of curcuminoids from C. zedoaria.

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