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Innovative Green Approach for Extraction of Piperine from Black Pepper Based on Response Surface Methodology

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Abstract: Bioactive compounds like piperine (alkaloids) offer a variety of health benefits due to their biological and pharmacological potential. Piperine has been revealed to have anti-inflammatory, anti-aging, anti-diabetes, anti-bacterial, anti-ulcer, and anti-carcinogenic characteristics. Recent research has been conducted to extract piperine using effective and environmentally friendly techniques. In this study, we sought to assess the potential and efficacy of natural deep eutectic solvents to extract piperine from black pepper seeds using an ultrasound-assisted extraction technique. A Box–Behnken design combined with response surface methodology was used to evaluate the optimum extraction conditions of piperine. Extraction efficiency was evaluated based on the extraction yields of piperine, antioxidant activity, total polyphenols, and total flavonoids. The results showed that the choline chloride-citric acid-1,2-propylene glycol combination (1:2:2 molar ratio) with 25% (*v*/*v*) of water was the most effective at extracting piperine from black pepper. It was found that the extraction yield of piperine was significantly influenced by the liquid–solid ratio and extraction time. The optimal extraction conditions were determined and it was found that antioxidant activities and total polyphenol content in the piperine-rich extracts were remarkably related to the piperine content. The piperine extract purity was found to be 90%. Our results indicate that black pepper could be used as a functional food application.

Keywords: *Piper nigrum* L.; piperine; eutectic solvent; flavonoids; polyphenols; response surface methodology

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1. Introduction

Piperine (PIP) is the most common plant alkaloid found in the Piperaceae species which has recently received a lot of attention as a result of its extensive array of advantageous biological and pharmacological features [1]. Piperine is a weakly basic, polar compound, defined as a yellow crystalline material having the molecular formula $C_{17}H_{19}NO_3$ (Figure 1) [2]. Piperine has been shown to have antioxidant, anti-microbial, anti-fungal [3,4], anti-diarrhoeal [5], anti-inflammatory [6,7], as well as anti-cancer [8] and immunomodulatory effects [9], neuroprotective, anti-parasitic, -analgesic, anti-inflammatory, hepatoprotective, and anti-aging effects [3,4].

Black pepper, being "the king of spices" [5,6] is a widely consumed spice that traditional medicine also used as an ingredient for physiological and pharmacological activities. Typically, piperine is eaten with black pepper or white pepper and helps enhance metabolism and nutrient adsorption. Black pepper has sparked increased interest due to its piperine content, thereby being one of the most widely used plants in the pharmaceutical industry [7]. PIP as a nutraceutical agent, according to sub-acute toxicity testing up to a

dose of 100 mg/kg, may be harmful to the body if ingested in high doses [4]. However, consuming raw black pepper is insufficient because it contains only about 3% to 9% piperine, resulting in a weaker effect.

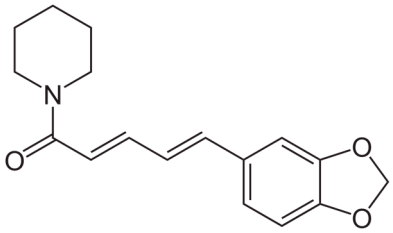

Figure 1. Chemical structure of piperine.

Natural deep eutectic solvent (NADES) is a novel type of eco-friendly solvent that shares some characteristics with ionic liquids and is formed by mixing a quaternary ammonium salt or hydrogen-bond acceptor (HBA) with a hydrogen-bond donor (HBD) [8,9]. NADES are considered one of the most effective green solvents [10] in terms of extractability, stability, and recovery yield, as well as their bioactivity, solubility, and bioavailability. Furthermore, NADES is cheap, affordable, easy to use, and less time-consuming. Moreover, NADES has been recognized to have minor harmful effects and is hence used to enhance the extraction efficiency of plant-derived bioactive compounds [11–14]. Since choline chloride is cheap and biodegradable, it has been widely used in previous literature as a main component of NADES [15,16].

Substantial efforts have been made to improve the extraction yield of natural bioactive compounds from plant material by combining NADES with innovative extraction techniques. These novel techniques include accelerated solvent extraction, ultrasound, microwave, supercritical, enzymatic, pressurized liquid, high-intensity pulsed current, and high hydrostatic pressure [13]. Among these techniques, ultrasound-assisted extraction (UAE) was utilized to overcome the drawbacks associated with conventional methods [17]. The cavitation phenomenon and thermal effects of UAE enhance the mass and cause better cell disruption [18]. It has been reported that using conventional solvents is hazardous mainly due to the fact that possible solvent residues may remain in the final extracts [11,19,20].

Extraction of bioactive compounds from seeds, e.g., polyphenols and flavonoids using NADES coupled with UAE has been reported [21–23]. The extraction, isolation, characterization, and biological properties of piperine from pepper fruits including *Piper nigrum* L. and *Piper longum* have also been studied [24–26]. This broad research has led to significant advances in the discovery of pharmacological activities with therapeutic potential and significant drug-like properties of piperine [27]. It was observed that the yield of extractability and its purity depend roughly on the solvent type and extraction technique.

Response surface methodology (RSM) is an effective tool that allows researchers to establish an optimization process by determining the response to multiple parameters [28]. In practice, the design of experiments (DOE) assists us to select the best model and optimum experimental conditions, e.g., response surface methodology (RSM), artificial neural network (ANN), support vector machine (SVM), and genetic algorithm (GA) [29,30]. While RSM is one of the most popular methodologies, Box–Behnken design (BBD), Doehlert matrix (DM), and central composite design (CCD) are considered the most prevalent designs used for the optimization of experimental parameters [31]. Compared to other RSMs, Box–Behnken design (BBD) is considered a convenient tool that is widely used for RSM experimental design which can simultaneously determine the correlation between different variables and optimizes their responses [32,33].

There has been only one report that described the use of 1-alkyl-3-methylimidazolium as an ionic liquid for ultrasonic-assisted extraction of piperine from white pepper and

optimized the extraction conditions using the univariate method. According to this study, only single parameters, e.g., ultrasonic power, extraction time, and solid–liquid ratio have been used without utilizing NADES or RSM for optimizing the extraction conditions [34]. Thus, there are no previous recorded findings on the use of RSM as an innovative approach for extracting piperine from black pepper.

This work aims to develop a novel NADES-based UAE to extract PIP from black pepper and optimize its yield using RSM. In view of this, we examined the extractability of 16 different NADES and compared their final yield to the conventional extraction methodologies. The efficacy of extraction was evaluated in terms of total polyphenol content and flavonoid content, as well as antioxidant activity.

2. Materials and Methods

2.1. Instrumentation

An Elma-ultrasonic cleaner P30H (Singen, Germany), a laboratory centrifuge PE-6926 (Sochi, Russia) and a rotary evaporator were utilized, and a spectrophotometer Shimadzu-UV VIS 2600 (Tokyo, Japan) was used for the analysis of PIP extract.

2.2. Chemicals and Reagents

2.2.1. Plant Material

Piper nigrum Linn. was purchased from the market and pulverized into a homogeneous powder using an electric blender. The powder was stored in a room environment until it was subjected to quantitative analysis.

2.2.2. Natural Deep Eutectic Solvents

To obtain black pepper powder, NADES were employed at a 15:1 material ratio of NADES to *Piper nigrum* Linn. in volume (mL)/weight (g). As shown in the table below, 16 NADES were used in different molar ratios in the current study. It should be noted that any solvents that did not remain stable at room temperature for 24 h (like the mixture L-Proline: urea, L-Proline: succinic acid, and choline chloride: succinic acid) were discarded from our experiment. Hence, some of the combinations were selected based on pilot experiments using different combinations and molar ratios (data not shown) or previous literature [35–38]. Table 1 elucidates all NADES components including HBA and HBD used in this work.

2.2.3. Other Reagents

Distilled water, ethanol (95%, 30%), Folin–Ciocalteu, Na_2CO_3 20%, gallic acid, Quercetin, 2,2-Diphenyl 1-PicryHydrazyl (DPPH), $NaNO_2$ 5%, $AlL(NO_3)_3$ 10%, NaOH 1M, ethyl acetate, and methanol were used as reagents for the extraction process and analysis of samples.

2.3. Isolation and Purification of Piperine

Utilizing a NADES-based UAE procedure, piperine was isolated from *Piper nigrum* L. Approximately 0.06 g of inert powder was precisely weighed into a 1.5 mL microcentrifuge tube (Eppendorf), which was immediately filled with 0.9 mL of NADES solution. An ultrasonic bath was used to process the mixture using ultrasonic vibrations at a frequency of 37 kHz and a power of 100 W at 35 °C for 30 min. PIP was extracted gradually into the NADES phase, resulting in a viscous suspension. To distinguish the solid from the liquid phases, the suspension was centrifuged for 10 min at 10,000 rpm using the laboratory centrifuge. The resulting liquid was fractionated using a separatory funnel with ethyl acetate, and a rotatory evaporator was used to concentrate the organic layer until it was dry. The solid extract was finally stored at −20 °C until further use. A portion of the final extract was weighed, then diluted in ethanol (95%), and centrifuged for another 5 min at 10,000 rpm. Piperine concentrations in the resulting solution were determined using a UV-Vis spectrophotometer, and their values were calculated by plotting a calibration

curve. All experimental treatments were performed in triplicate using the same procedure. Deionized water, 80% of aqueous methanol, and ethanol were used as control extractions under identical conditions.

Table 1. NADES components used for extraction of piperine from *Piper nigrum*.

	NADES Components			
	HBA *	**HBD ****	**HBD ****	**HBA:HBD:HBD Ratio**
1	Choline Chloride	Urea	-	1:1
2	Choline Chloride	1-2 Propylene glycol	-	1:1
3	Choline Chloride	Malic Acid	-	1:1
4	Choline Chloride	Citric Acid	-	1:1
5	L-Proline	Malic Acid	-	1:1
6	L-Proline	Citric Acid	-	1:1
7	Choline Chloride	Glycerin	Urea	1:1:1
8	L-Proline	Glycerin	Malic Acid	1:2:2
9	Choline Chloride	1-2 Propylene Glycol	Citric Acid	1:3:1
10	Choline Chloride	Urea	Citric Acid	1:2:1
11	Choline Chloride	Malic Acid	Citric Acid	1:1:1
12	Choline Chloride	Glucose	Citric Acid	1:1:1
13	Choline Chloride	1-2 Propylene Glycol	Citric Acid	1:2:2
14	Choline Chloride	Glycerin	Citric Acid	1:2:2
15	Choline Chloride	Urea	Citric Acid	1:2:2
16	Choline Chloride	Malic Acid	Citric Acid	1:2:2

* Refers to hydrogen bond acceptor. ** Refers to hydrogen bond donor.

2.4. Piperine Yield and Experimental Design

2.4.1. Piperine Yield

PIP was quantified using the calibration curve generated by spectrophotometric analysis. Absorption of piperine in the UV range at 345 nm was used as the basis for the UV spectrophotometric method. For quantification, a calibration curve was used with piperine pure solutions (98%) in ethanol at concentrations varying from 1 to 16 µg/L (Figure 2).

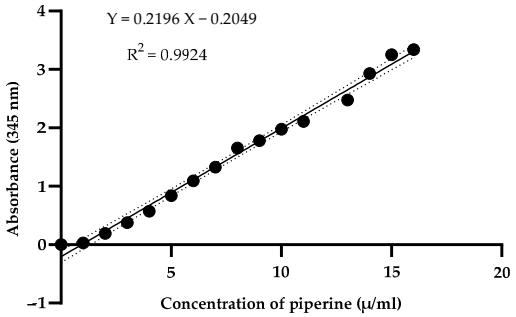

Figure 2. The standard curve of piperine by UV spectrophotometry.

The yield of PIP was determined according to the following equation:

$$\text{Yield} = \frac{\text{Cp} \times \text{Vs}}{\text{Ms}} \quad (1)$$

where Cp is the concentration of piperine found in the dry extract powder based on spectrophotometric analysis, Vs denotes the diluted suspension volume, and Ms the mass of the test sample (black pepper powder).

2.4.2. Experimental Design

The best NADES mixture was selected for further optimization of the extraction conditions prior to using RSM. The ideal values from the four extraction variables including

time (A), temperature (B), water content within NADES (C), and solid–liquid ratio (D) at three levels (−1, 0, +1) were using RSM based on a Box–Behnken design (BBD).

The following extraction parameters were set:

- Extraction time (A): 20–60 min;
- Extraction temperature (B): 25–60 °C;
- Water percent (C): 10–30%;
- Solid–liquid ratio (D): 10–40 mL/g.

Box–Behnken design.

Other in vitro parameters such as total polyphenol content (TPC), antioxidant activity (AOA), and total flavonoid content (TFC) were evaluated using samples obtained from the optimized and conventional extraction conditions. The purity of our best extract was found to be 0.90%. The purity was assessed using the following formula:

$$\text{Purity} = \text{Yield}/(\text{mass solid extract}/\text{total mass of sample}) \quad (2)$$

2.5. Evaluation of Extraction

2.5.1. Antioxidant Activity (AOA)

Using a modified Ozturk et al. [39] technique, the capacity to neutralize the "stable" free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was assessed. In a nutshell, 100 µL of the extract was diluted ten times, and 0.4 mL of the diluted plant extracts were combined with 3.6 mL of DPPH ethanolic solution (0.1 mM). An equivalent volume of ethanol (0.4 mL) was used as a blank (control) with 3.6 mL of the DPPH solution. Before incubating at 37 °C for 30 min in the dark, all samples were prepared in triplicate and vortexed for 1 min. On a UV–Visible spectrophotometer set to 517 nm, the decrease in absorbance of each sample was measured against ethanol as a blank. Percentage DPPH inhibition was calculated using the formula below:

$$\text{Inhibition } (\%) = \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \times 100 \quad (3)$$

where the only ingredients in the control were ethanol and DPPH solution. The values were presented as the mean standard deviation for all the reactions, which were all monitored in duplicate (SD).

2.5.2. Total Polyphenol Content (TPC)

A slightly modified version of the previously reported approach was used to calculate TPC [40]. Samples (0.25 mL) and reference gallic acid solutions (0, 50, 100, 150, 250, and 500 mg/L) were pipetted into test tubes. In order to homogenize the Folin–Ciocalteu solution, distilled water (5.5 mL) was added.

After 5 min of incubation,1 mL of Na_2CO_3 (20%) was added. Test tubes were incubated at 20 °C for two hours, and the absorbance was predicted using a UV-Vis spectrophotometer (UV-2600, Tokyo, Japan) at 765 nm in 30 min against a blank (distilled water). TPC was calculated using the equation generated from the calibration curve expressed as mmol/L of garlic acid equivalents per g of extract:

$$Y = 0.0038X + 0.0487 \ (R^2 = 0.9982) \quad (4)$$

2.5.3. Total Flavonoid Content (TFC)

Huang et al. [41] described the $NaNO_2$–Al $(NO_3)_3$–NaOH colorimetric method, which was followed with minor changes. Briefly, 0.15 mL of $NaNO_2$ (5%, *w*/*v*), 2 mL of 30% ethanol, and 0.5 mL of piperine extract were combined. The combination underwent a 6-min reaction with 0.15 mL of Al $(NO_3)_3$ (10%, *w*/*v*) after 5 min of reaction. After that, 10 mL of the mixture was obtained by adding 0.1 mL of 30% ethanol and 2 mL of 1 M NaOH. At 510 nm, the absorbance was measured following a 10-minute incubation at room temperature. In the blank, the same amount of 30% ethanol was used in place of

the $Al(NO_3)_3$ and NaOH solutions. The range of the calibration curve, which was used to express the total flavonoid concentration of the samples in mmol/L quercetin equivalent was 0 to 500 g/mL.

2.6. Statistical Analysis

The one-way analysis of variance (ANOVA) and Tukey's and Sidak's significance tests were performed using the statistical analysis program GraphPad Prism 9.4.1. p values under 0.05 were regarded as significant. Correlations between variables were evaluated using Pearson's correlation approach at a significance threshold of $p \leq 0.01$. All through the Design expert software 13.0.5.0, the optimum conditions (parameters) for extracting Piperine were discovered. The means and standard error are used to express the data in the text and tables.

3. Results and Discussion

3.1. Evaluation of Piperine Using NADES Extraction

In the current work, the efficacy of piperine extraction was tested using 16 NADESs derived from a single group of HBAs (choline chlorine and L-proline) in combination with 6 types of HBDs (urea, 1-2 propylene glycol, malic acid, citric acid, glycerin, and glucose). All NADESs created from these solid and liquid starting materials were examined to be stable as an optically clear fluid at room temperature. It was previously reported that the variability and dependency of the viscosity of these NADESs are significantly influenced by the intermolecular interactions between HBDs and HBAs [42]. The NADESs have higher viscosities than conventional solvents due to the hydrogen bond, Van der Waals, and electrostatic interactions between the components, which constrict their usage in the extraction of bioactive compounds due to mass transfer delay [43]. As a result, all the NADESs were screened as a mixture made by mixing the NADES with additional water in a 25% (v/v) ratio. The yields of piperine were determined in order to select the most effective NADES for piperine extraction. We used a UV-Vis spectrophotometer to evaluate the yield of piperine, which is one of the most abundant bioactive compounds in black pepper, with absorbance at 345 nm [26,44].

For a more accurate measurement, the calibration curve was plotted using 16 different concentrations of pure piperine (98%). Figure 3 summarized the extraction yield results of piperine from black pepper samples using different NADES and different conventional solvents. As shown in Figure 3, NADES-13 had the highest extraction yield value of piperine (38.76 mg/g). The one-way ANOVA with Sidak's multiple comparisons analysis (95.00 percent of diff) between the target NADES and others was used to show the significance of difference among values, as indicated by one or multiple asterisks. Figure 3 revealed a noticed significant difference in PIP extractability using different types of NADES. When compared to NADES-6, some NADES extraction yield values were found to be non-significant (ns), whereas when compared to NADES-13, all NADES showed significant differences ($p < 0.05$).

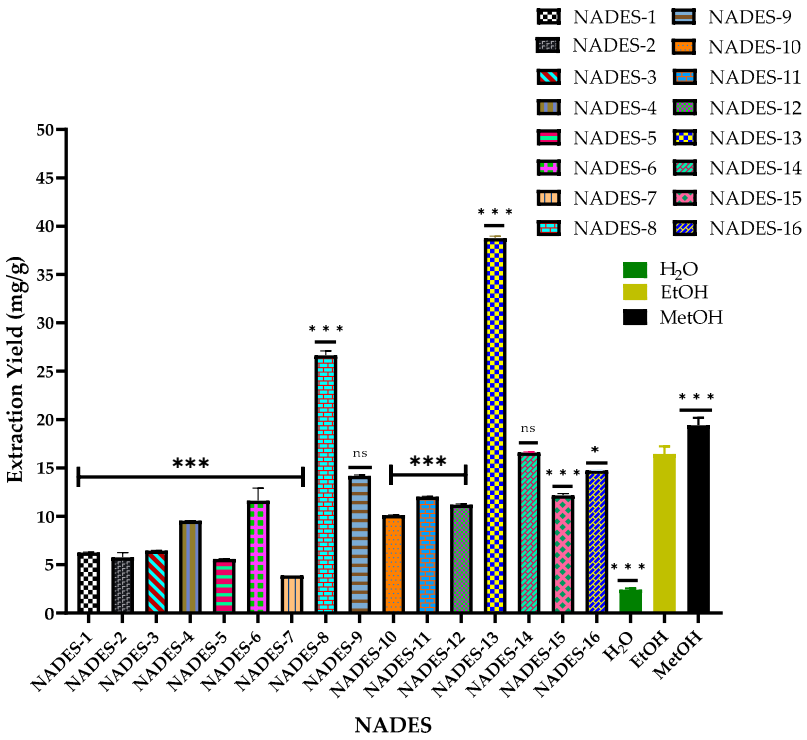

Figure 3. Extraction yield (mg/g) of NADESs and three conventional solvents. Black bars and asterisks refer to the degree of significance of all solvents against ethanol. One-way ANOVA was used to analyze the data, followed by Sidak's multiple comparisons.

3.2. Optimizing the Extraction Conditions by Experimental Design

3.2.1. Design of Experiment Methodology and Factor Signification

Using three levels of each independent variable, including extraction time, temperature, water content, solid–liquid ratio, and extraction yield, RSM experiments were employed to effectively optimize the extraction yield of piperine. To determine the efficiency of the extraction technique, the extraction yield was referred to as the response. To prevent any systematic error, the trials were all carried out in random order. Table 2 displays the findings, together with the coded factors and pertinent responses. After performing several regression analyses on the experimental data, a second-order polynomial equation was used to express the suggested model for analyzing the results. The following is the regression model equation for the response and variables using the coded levels:

$$\begin{aligned} Y_p = 31.97 + \; & 4.37A + 2.11B - 1.53C + 8.94D - 2.79AB \\ & -0.4155AC + 4.17AD - 0.0740BC + 0.2539BD \\ & +0.7001CD + 0.6504A^2 + 1.56B^2 - 1.34C^2 \\ & -0.5888D^2 \end{aligned} \quad (5)$$

where (Y_p) is the yield of piperine, (A) extraction time, (B) extraction temperature, (C) water content in NADES, and (D) solid–liquid ratio.

Table 2. Results of the response surface experiment (Box–Behnken design) for optimization of the independent variables used in deep eutectic solvent extraction of piperine from *Piper nigrum* seeds.

	Coded Variables				Variables				Results	
	A	**B**	**C**	**D**	**Extraction Time (min) (A)**	**Extraction Temperature (°C) (B)**	**Water Content (%) (C)**	**Solid–Liquid Ratio (mg^{-1}) (D)**	**Yield (mg/g)**	**SD**
1	0	1	−1	0	40	45	20	25	36.23	1.16
2	0	1	0	−1	20	45	10	25	31.10	0.46
3	1	0	1	0	40	60	10	25	37.6	3.43
4	−1	1	0	0	60	45	20	10	22.28	1.11
5	1	1	0	0	20	60	20	25	28.63	3.08
6	0	−1	1	0	40	45	10	10	25.61	1.01
7	1	0	0	−1	40	45	10	40	40.31	0.67
8	0	0	−1	1	20	30	20	25	24.44	0.16
9	0	0	0	0	20	45	20	10	22.96	2.52
10	0	−1	0	−1	40	30	30	25	27.28	0.91
11	0	0	1	−1	20	45	30	25	27.87	1.27
12	0	0	0	0	40	30	20	40	38.16	1.31
13	1	0	0	1	40	60	20	40	43.72	2.10
14	−1	0	0	1	40	45	20	25	32.29	0.08
15	0	−1	−1	0	40	45	20	25	28.34	4.04
16	1	0	−1	0	20	45	20	40	33.81	1.77
17	0	0	0	0	40	60	20	10	24.85	0.50
18	−1	0	−1	0	40	45	20	25	36.89	0.30
19	0	0	−1	−1	40	30	10	25	28.45	1.78
20	0	0	0	0	40	45	30	40	37.91	0.81
21	−1	0	0	−1	60	45	10	25	33.16	2.56
22	1	−1	0	0	40	30	20	10	20.31	0.91
23	0	−1	0	1	60	60	20	25	40.36	2.02
24	0	1	1	0	40	60	30	25	36.13	2.27
25	0	0	0	0	40	45	30	10	20.40	2.22
26	0	0	1	1	40	45	20	25	30.57	0.08
27	0	1	0	1	60	45	30	25	28.27	5.81
28	−1	−1	0	0	60	45	20	40	49.80	0.20
29	0	0	0	0	60	30	20	25	47.35	0.86
30	−1	0	1	0	40	45	20	25	27.55	0.13

The square of the correlation coefficient (R^2) was used to evaluate the validity of the model, and an ANOVA with a 95% confidence level was used to evaluate the lack of fit. Piperine response's coefficient of determination (R^2) was found to be 0.8243 (Table S1). Our assumption that the model was enough to accurately reflect the experimental data was reinforced by the F-value for the lack-of-fit model being non-significant.

3.2.2. Determination of the Optimal Conditions by RSM

The interactions between piperine yields of all runs were evaluated using a three-dimensional (3D) response surface plot of the model. The extraction yield of piperine appears to vary depending on the differences between the four independent variables. As shown in Table 2, the liquid–solid ratio (D) and extraction time (A) were both statistically significant in this model ($p \leq 0.05$), although the concentration of water (%) in the NADES (C) and the extraction temperature (B) did not exhibit a significant relationship ($p > 0.05$). It was also found that the yield increased rapidly as extraction time and liquid–solid ratio values increased. The maximum extraction yield of piperine was noted at a liquid–solid ratio value of 40, as illustrated in Figure 4C. The extraction yield was also at its peak at 60 min. In general, time is a critical factor in optimizing process development, and lowering energy and cost.

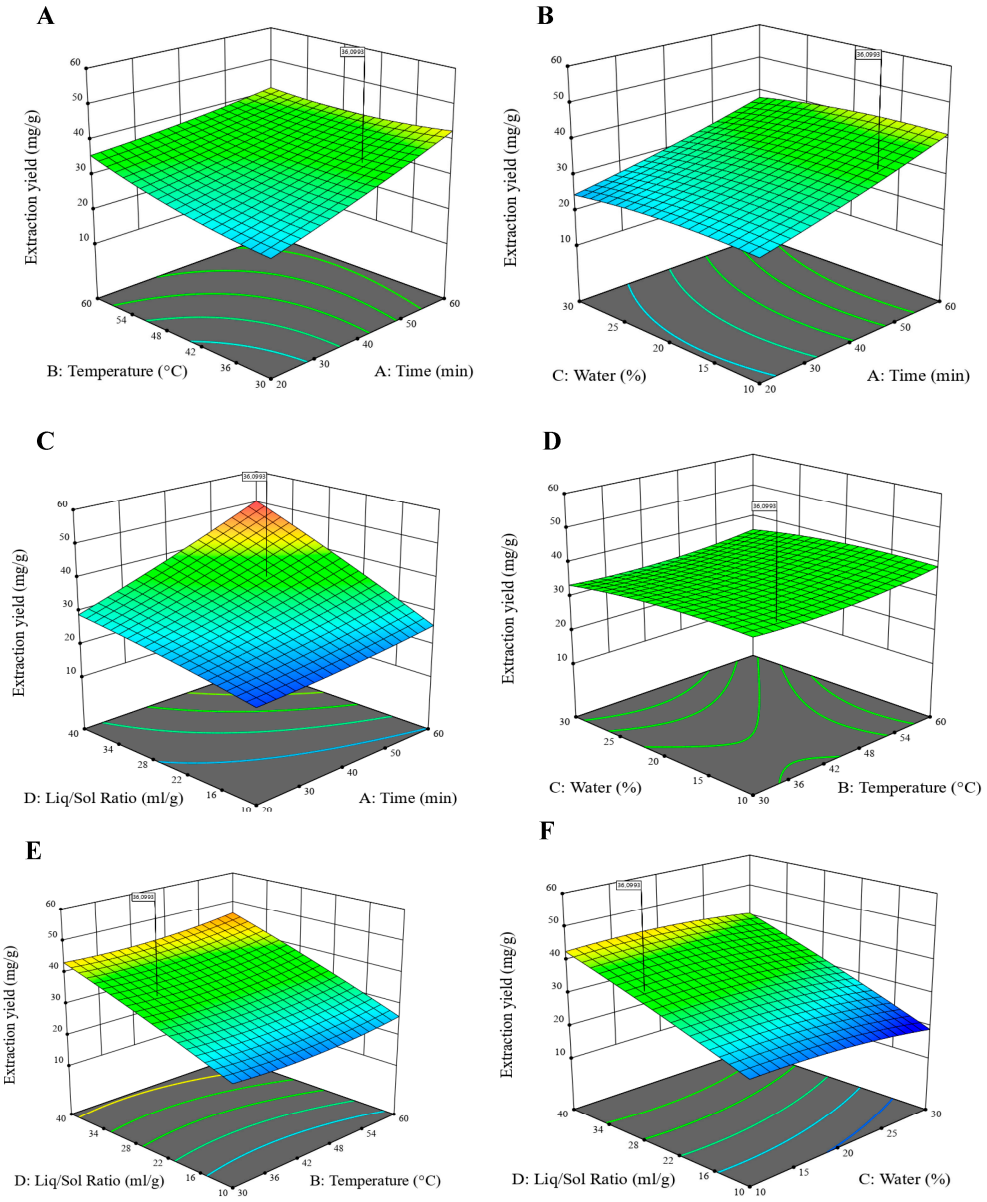

Figure 4. Response surfaces of piperine content for optimization. (**A**) Yield vs. water % and temperature; (**B**) Yield vs. water % and time; (**C**) Yield vs. Liq/sol and time; (**D**) Yield vs. water % and temperature; (**E**) Yield vs. Liq/sol and temperature; (**F**) Yield vs. Liq/sol and water %.

The increase in water percentage, on the other hand, had a negative effect on the extraction yield, as shown in Figure 4D. When the water percentage in NADES was at its highest, as shown in Figure 3, piperine extraction dropped to its lowest level. Adding water reduces HBA basicity while increasing polarizability and bipolarity. When NADESs have enough water, their viscosity and polarity vary, allowing researchers to modify their characteristics and make them appropriate extraction mediums for undesirable chemicals. Low extraction yields were explained by the weakened interactions between NADES and the targeted compound which occurred after the addition of 50% (v/v) water, according to Dai et al. and others [45–48]. The viscosities of the NADESs were also found to be extremely temperature sensitive. Meanwhile, the temperature's positive effect on piperine extraction efficiency appeared to be due to its effect on diffusion and solubility rates. In this experiment, however, piperine yield was particularly more affected by the solid–liquid ratio and extraction time than other factors. Piperine yield was the highest when they were at their peak (60 min, liquid–solid ratio 40).

The temperature had a favorable impact on extraction yield whereas the greatest value was at 45 °C and diminished as the temperature rose. It is well-known that temperature influences diffusion and solubility rates, therefore, the viscosities of the NADESs were found to be temperature sensitive. The temperature effect on the extraction yield of piperine was, however, less noticeable in the model than the other three factors shown in Figure 4E. Further temperature increases, however, would not result in a rise in extraction yield due to the lower level of cavitation [49]. Finally, we utilized a simple procedure to evaluate the optimal extraction conditions of piperine from black pepper, as well as the related maximal response values elucidated in Table 2. Piperine extraction yields were highest at the following conditions: extraction time 60 min, temperature 45 °C, 20% water, and a liquid–solid ratio of 40 mL/g. While RSM recommended a 50-min extraction time, a temperature of 30 °C, a water content of 14.5% in NADES, and a solid–liquid ratio of 30 mL/g yielding 39.07 mg/g as the ideal conditions (Table 3).

Table 3. Optimum conditions of the variables that maximize the response values using RSM.

Parameters	A (min)	B (°C)	C (%)	D (mg^{-1})	Optimum (mg/g)	Desirability
Values	50	30	14.5	30	39.075	0.923

Table 3 provides results related to the optimum conditions of factors maximizing the response values using the RSM. The obtained results using NADES extraction were similar to the data published by Gorgani et al. [24], whereas the extraction yield of piperine was 38.8 mg/g and 39.1 mg/g after utilizing microwave-assisted or Soxhlet extraction technologies, respectively. Despite the slightly lower concentration of our optimum extraction yield of piperine than that obtained by ethanol (46.6 mg), we assume that eco-friendly extraction technology using NADES is more efficient since no organic solvents were used to extract piperine [24]. Nevertheless, our optimum extraction yield of piperine (39.075 mg/g) was higher than previously reported data (35.77 mg/g; 5.64 mg/g) by Cao et al. [34] and Olalere et al. [25]. This could be explained by the efficiency of NADES to extract piperine and the practicability of RSM to optimize the employed extraction conditions.

3.3. Antioxidant, Total Polyphenols, and Total Flavonoids

Although UV-Vis spectrophotometric analysis is well-known for its wide use in experimental research, there could be some unknown organic or inorganic substances in the final extracts of piperine that may interfere with the color formation, and an absorbance peak of samples cannot be clearly distinguished. Therefore, we attempted to recover piperine from NADES using ethyl acetate and hence eliminating the interfering substances and minimizing the error. Moreover, all experimental analysis has been conducted in triplicates in order to increase the precision and accuracy of the results.

As shown in Table 4, the piperine-rich NADES extract showed the highest inhibition percent (45.34%) in the DPPH assay followed by methanol (22.66 ± 0.85%) and ethanol (21.60 ± 2.47%), while piperine-rich H_2O extract exhibited the lowest result. Previous authors reported the antioxidant activity of piperine such as inhibiting or scavenging both free and hydroxyl radicals and ROS [50,51]. These outcomes are in agreement with the previously published results where the scavenging activity was piperine-concentration dependent [52].

Table 4. Comparison of the extraction efficiencies of NADES (optimum result) with conventional solvents.

	AOA (%)	TPC (mmol/L)	TFC (mmol/L)
H_2O	11.81 ± 1.61	3.95 ± 9.31	1.22 ± 0.11
EtOH	21.60 ± 2.47	49.42 ± 7.44	40.57 ± 4.04
MeOH	22.66 ± 0.85	46.06 ± 11.17	39.15 ± 6.06
NADES-13	45.34 ± 5.78	94.876 ± 1.86	82.033 ± 9.09

H_2O, Water; EtOH, ethanol; MeOH, methanol.

Total flavonoid and total polyphenol contents showed a similar pattern. The highest extraction efficiencies resulted in a piperine extract NADES-13, while it was noticed that piperine-rich H_2O extract exhibited the lowest results for all three parameters. This could be explained by the lower piperine content in water extract compared to ethanol and methanol extracts. Generally, the total polyphenol content and antioxidant activities of the piperine-rich extract were found highly attributed to the piperine content. Moreover, it was previously stated that antioxidant activity is largely associated with total polyphenol content [53]. The results showed that decreasing the polarity of the solvent used resulted in the highest content of flavonoid and polyphenol content. The discrepancies in the polyphenol and flavonoid components of the various extracts seen in some other investigations could be explained by the polarity of chemicals contained in the raw material [52].

Pearson correlation analysis between AOA, TPC, and TFC was carried out. The antioxidant activity showed a high Pearson correlation coefficient (p (two-tailed) ≤ 0.01) between TPC values and antioxidant activity, indicating the presence of active piperine, as polyphenol-derived alkaloids, in the NADES solution may significantly enhance DPPH free radical scavenging activity. These outcomes are in agreement with the previous investigations where a positive correlation was found between antioxidant and total phenolic [53]. In contrast, there was no correlation found between the TFC and the other parameters among the tested extracts. Importantly, there is not a general and definite relationship between TPC and TFC but, theoretically, it is well-known that TPC must always be greater than TFC because flavonoids (rutin, quercetin, etc.) are a type of polyphenol such as (vanillin, catechin, gallic acid, etc.). However, only in very few studies was the TFC found to be greater than the TPC depending on the extraction methods [54]. It is somewhat evident that TPC determined using the Folin–Ciocalteu procedure does not fully reflect the quality of polyphenolic compounds in extracts [55]. Overall, the present study confirmed that black pepper contains a considerable amount of polyphenols and flavonoids that may possess therapeutic potential.

4. Conclusions

In this work, piperine was extracted from black pepper by utilizing a novel green extraction technique based on natural deep eutectic solvents and optimized using RSM. Compared to alternative hazardous conventional procedures, NADES combined with ultrasonic-assisted extraction is an effective solvent for the extraction of piperine. The findings of the solvent screening revealed that NADES containing choline chloride, 1-2 propylene glycol, and citric acid in a molar ratio of 1:2:2 was the most effective for extracting piperine from black pepper. In general, the optimum piperine yield obtained by RSM was considerably high compared to that obtained by the other innovative extraction technologies. The antioxidant activity was shown to be the highest in the piperine extract obtained by NADES and it was significantly correlated to total polyphenols in the extracts. We anticipate that the findings of this study could contribute to establishing black pepper as a resource for natural bioactive piperine and promote the consumption of these spices as a functional food ingredient and source of nutraceutical and pharmaceutical compounds.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/suschem4010005/s1, Table S1: ANOVA results for the established model of piperine.

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