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Development and optimization of *Clinacanthus nutans* liquid spray using a two-level full factorial design

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This study aimed to develop and optimize a topical *Clinacanthus nutans* liquid spray that dries quickly after spraying and has low stickiness. An experimental design was adopted to evaluate the effects of the amounts of polyvinylpyrrolidone (X_1) and glycerol (X_2) and the ratio of acetone:ethanol (X_3) on the stickiness (Y_1), solution viscosity (Y_2), evaporation rate (Y_3), and spray coverage radius (Y_4) of the solution. Results revealed that all independent factors had a substantial effect on Y_1–Y_3. In this model, the synergistic effect was uncommon. The optimized formulation consisted of X_1 of 0.1%, X_2 of 1.0%, and X_3 of 59:41. The response values of Y_1, Y_2, Y_3, and Y_4 were 92.24 mN, 4.63 cP, 0.0959%, and 9.40 cm, respectively. The actual results were close to those predicted by the experimental design model. Therefore, the optimized topical spray formulation can be used in actual practice.

1. Introduction

Clinacanthus nutans (Burm.f.) Lindau, also known as snake grass, is a member of the Acanthaceae family and has diverse and potential medicinal uses in traditional herb for treating skin rashes, insect and snake bites, lesions caused by herpes simplex virus, diabetes, and gout in many Asian countries, such as Malaysia, Indonesia, Thailand, and China (Alam et al. 2016; Zulkipli et al. 2017). This grass is one of the most well-known medicinal plants listed in Thai Herbal Pharmacopoeia with anti-inflammatory and antiviral activities. Its major phytochemical bioactive compounds are flavonoids, glycosides, glycoglycerolipids, cerebrosides, and monoacylmonogalatosyl glycerol (Alam et al. 2016). Its extracts and pure compounds exhibit a broad range of biological properties, such as anti-inflammatory, antiviral, antioxidant, and anti-diabetic activities (Alam et al. 2016; Zulkipli et al. 2017; Kamarudin et al. 2017; Phung et al. 2021). Owing to their lack of toxicity, these extracts can be used as a strong therapeutic agent for specific diseases. *C. nutans* extracts are generally formulated as oily ointment, balm, or cream (Sekar and Rashid 2016), all of which are not convenient for topical application and not suitable for hairy skin areas. Therefore, a topical liquid spray formulation has been developed with the following major ingredients: active substance, film-forming agent, and plasticizer (Umar et al. 2020). Other excipients such as humectant and antioxidant are also vital to obtain the desirable product. The properties of the prepared spray solutions, such as solution viscosity, drying time after spraying, skin moisture, and radius of spray coverage, must also be monitored.

Design of experiment (DOE) is necessary for optimizing any topical liquid spray formulation in a short period of time. DOE aims to describe or explain information variations under hypothesized conditions to reflect their differences (Cawse 2003). Several models of DOE have been invented and applied for various types of experiment (Tye 2004). Among these, a full factorial DOE can measure the response of every possible combination of factors and factor levels (Shah and Pathak 2010; Piriyaprasarth and Sriamornsak 2011). Moreover, the partial least squares regression (PLS) equations derived from the experimental results can reveal the relationship among responses and main/interacting effects (Mehmood et al. 2020). PLS is a tool for building predictive models where highly collinear factor and linear regression is ineffective due to the large number of variables and ill-understood relationships. With the use of these PLS models, the optimized formulation can be calculated under constraint and product requirement (Huanbutta et al. 2013).

This study aimed to develop a *C. nutans* topical liquid spray formulation by using a two-level full factorial design. Three independent variables of the formulation, namely, the amounts of polyvinylpyrrolidone (PVP) K30 and glycerol and the ratio of the system solvent (acetone:ethanol) were varied at two levels. Several dependent variables, namely, stickiness, spray coverage radius, solution viscosity, and evaporation rate, were monitored from all designed experimental runs. The DOE results were analyzed to obtain the polynomial models, which were then employed to establish the optimized formulation with the highest desirability. Finally, the properties of the predicted formulation were compared with those of the actual prepared formulation to examine the potential of the DOE models.

2. Investigations and results

2.1. Compound identification and flavonoid quantification from the extracts

Flavonoids are the largest group of naturally occurring phenolic compounds in different plant parts that can either appear in free state or as glycosides. In this study, the compounds in the extracts were identified by ferric chloride test, cyanidin test, and Molish's test. The results and interpretation of the tests are displayed in Table 1. According to the quantification test, the total flavonoid content of the extracts measured by UV-visible spectrophotometer was 531.1 μg/mL.

Table 1: Compound identification test of *Clinacanthus nutans* extract

Test	Result	Interpretation
Ferric chloride test	Dark brown solution	Presence of phenolic compound
Cyanidin test	Solution color turned to red-brown	Presence of flavonoid
Molish's test	A purple ring at the top of the solution	Presence of glycoside

2.2. Results of two-level full factorial design experiment

The results from the two-level full factorial design experiment are shown in Table 2 and were analyzed by the Design Expert® program (version 11). A PLS models were then generated and is presented in Table 3. All *p*-values for the PLS models were less than 0.05, indicating the significant effect of the factors (X) on the responses (Y). Moreover, the adjusted and predicted R^2 values were 85.30%–99.99%, implying the high reliability of the models.

2.2.1. Stickiness

The PLS model of stickiness was significant with its *p*-value of <0.05 (Table 3). The adjusted and predicted R^2 values were 99.99%, indicating that the was well-fitted to the original data. All factors significantly affected the stickiness of the spray solution as shown in Table 4A. the amount of PVP K30 and glycerol substantially increased the stickiness of the formulation as illustrated in Figure 1B.

2.2.2. Viscosity

The viscosity of all formulations in this study is shown in Table 2. As presented in Table 3, the PLS model of viscosity was significant ($p < 0.05$), indicating statistical relation between the response and the factors. The adjust R^2 was more than 90%, implying that the selected terms can improve the model better than what would be expected by chance. As shown in Table 4B, only main factors (X_1, X_2, and X_3) significantly affected the viscosity of the solution.

Table 2: Evaluation results (mean±standard deviation, n = 3) from the 2-level full factorial design experiment

Factor			Stickiness (mN) (Y_1)	Viscosity (cP) (Y_2)	Evaporation rate (% difference) (Y_3)	Radius of spray coverage (cm) (Y_4)
X_1	X_2	X_3				
-	-	-	104.12±0.73	4.54±0.40	0.0742±0.0038	9.60±0.06
+	-	-	401.78±5.53	28.28±7.71	0.0584±0.0012	6.14±0.47
-	+	-	182.11±0.36	8.18±1.46	0.0654±0.0051	7.65±0.35
+	+	-	983.94±5.58	28.64±1.91	0.0648±0.019	7.79±0.34
-	-	+	85.83±0.73	3.21±0.05	0.1076±0.0017	9.30±0.54
+	-	+	557.65±3.19	15.06±0.19	0.0725±0.0006	6.72±0.36
-	+	+	157.37±1.23	4.68±0.18	0.0938±0.0007	8.25±0.42
+	+	+	901.84±2.46	25.09±0.20	0.0535-.0048	6.26±0.25

Table 3: PLS model of different response variables

Response variable	PLS model	*p*-value of model	Adjusted R^2	Predicted R^2
Stickiness (Y_1)	$Y_1 = 289.47X_1+134.48X_2+3.842X_3+97.10X_1X_2+ 14.60X_1X_3-30.55X_2X_3 -28.94X_1X_2X_3-421.83$	0.000	99.99%	99.99%
Viscosity (Y_2)	$Y_2 = 9.22X_1+1.60X_2-3.04X_3+15.05$	0.000	90.61%	85.30%
Evaporation rate (Y_3)	$Y_3 = -0.01X_1-0.004X_2+0.01X_3-0.01X_1X_3-0.004X_2X_3-0.002X_1X_2X_3+0.07$	0.000	94.70%	91.71%
Radius of spray coverage (Y_4)	$Y_5 = -0.99X_1-0.28X_2+0.52X_1X_2-0.38X_1X_2X_3+7.71$	0.000	91.34%	86.44%

* Coefficient values in the model are in unit of the experimental design code.

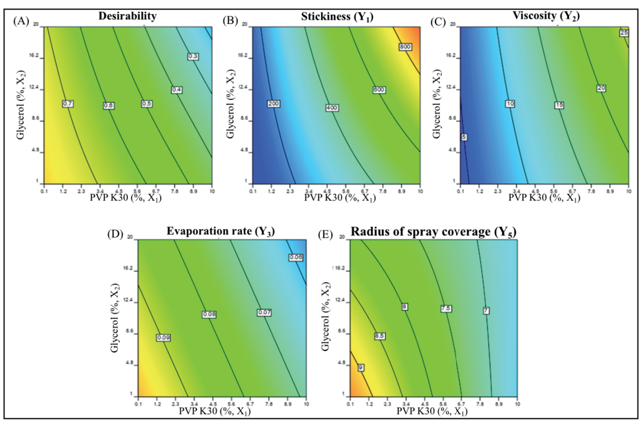

Fig. 1: Corresponding contour plots showing the influence of PVP K30 (X_1) and glycerol (X_2) on the value of (A) desirability, (B) stickiness (Y_1), (C) viscosity (Y_2), (D) evaporation rate (Y_3), (E) radius of spray coverage (Y_4). The purple tint denotes a low value, whereas the red color denotes a high value or level.

2.2.3. Evaporation rate

The evaporation rate of the formulation was examined from the weight difference between the initial and after 5-min evaporation of the formulation. Table 4C shows that the main factors and most two-way interactions significantly affected the evaporation time. Only the interaction between PVP K30 and glycerol did not significantly affect the model. As shown in Table 3 (evaporation rate; Y_3) and Fig. 1D, PVP K30 was the major factor that retarded the evaporation period. Meanwhile, a high portion of acetone accelerated the evaporation rate. In addition to altering the evaporation rate, the excessive use of acetone can cause skin irritation and dryness. Therefore, the amount of acetone in the spray formulation's solvent was limited to 80%.

2.2.4. Radius of spray coverage

Table 4D shows that only acetone did not affect the size of the spray coverage. As presented in Table 3 and Fig. 1E, the high portion of PVP K30 and glycerol reduced the radius of spray coverage.

Table 4: ANOVA tables from PLS model

(a) Stickiness

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Model	7	2719392	338485	38416.49	0.000
Linear	3	2445484	815151	30609.72	0.000
PVP K 30	1	2011072	2011072	198871.04	0.000
Glycerol	1	434057	434057	42923.09	0.000
Acetone	1	354	354	35.04	0.000
2-Way Interactions		253807	84602	8366.16	0.000
PVP K 30*Glycerol	1	226288	226238	22377.19	0.000
PVP K 30*Acetone	1	5116	5116	505.96	0.000
Glycerol*Acetone	1	22402	22402	2215.33	0.000
3-Way Interactions	1	20101	20101	1987.30	0.000
PVP K 30*Glycerol*Acetone	1	20101	20101	1987.30	0.000
Error	16	162	10		

(b) Viscosity

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Mode	7	2448.59	349.30	32.69	0.000
Linear	3	2322.40	774.13	72.34	0.000
PVP K 30	1	2039.46	2039.46	190.59	0.000
Glycerol	1	61.38	61.38	5.74	0.029
Acetone	1	221.56	221.56	20.70	0.000
2-Way Interactions	3	94.96	31.65	2.96	0.064
PVP K 30*Glycerol	1	23.38	23.38	2.23	0.155
PVP K 30*Acetone	1	32.02	32.02	2.99	0.103
Glycerol*Acetone	1	39.07	39.07	3.65	0.074
3-Way Interactions	1	31.24	31.24	2.92	0.107
PVP K 30*Glycerol*Acetone	1	31.24	31.24	2.92	0.107
Error	16	171.22	10.70		

(c) Evaporation time

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Mode	7	0.007035	0.001005	59.75	0.000
Linear	3	0.005187	0.001729	102.81	0.000
PVF K 30	1	0.003153	0.003153	187.49	0.000
Glycerol	1	0.000467	0.000467	27.78	0.000
Acetone	1	0.001567	0.001567	93.15	0.000
2-Way Interactions	3	0.001693	0.000564	33.55	0.000
PVP K 30*Glycerol	1	0.000037	0.000037	2.21	0.156
PVP K 30*Acetone	1	0.001307	0.001307	77.70	0.000
Glycerol*Acetone	1	0.000349	0.000349	20.74	0.000
3-Way Interactions	1	0.000155	0.000155	9.19	0.008
PVP K 30*Glycerol*Acetone	1	0.000155	0.000155	9.19	0.008
Error	16	0.000269	0.000017		

(d) Radius of spray

Source	DF	Adj SS	Adj MS	F-Value	P-Value
Mode	7	2.10603	0.300861	8.64	0.000
Linear	3	0.81731	0.272437	7.32	0.002
PVP K 30	1	0.22234	0.222337	6.38	0.022
Glycerol	1	0.54904	0.549037	15.76	0.001
Acetone	1	0.04594	0.045938	1.32	0.268
2-Way Interactions	3	0.32471	0 108237	3.11	0.056
PVP K 30*Glycerol	1	0.27520	0.275204	7.90	0.013
PVP K 30*Acetone	1	0.00000	0.000004	0.00	0.991
Glycerol*Acetone	1	0.04950	0.049504	1.42	0.251
3-Way Interactions	1	0.96400	0.964004	27.68	0.000
PVP K 30*Glycerol*Acetone	1	0.96400	0.964004	27.68	0.000
Error	16	0.55727	0.034829		

2.3. *Optimization*

All the PLS models from different response variables were used to calculate the optimized formula (desirability value of 0.798) as follows: 0.1%, 1.0%, and 59:41 for the amounts of PVP K30 and glycerol and the acetone:ethanol ratio, respectively. After the optimized solution was obtained, the calculated formula was prepared and evaluated. The prepared product from the optimized formulation had a clear general physical appearance, mild flavor, and low viscosity. As shown in Table 5, the stickiness (92.24 mN), viscosity (4.63 cP), and evaporation rate (0.0915%) were as low as recommended for topical spray formulations. The radius of spray coverage was maximized, and the experimental results were higher than the predicted values. The product requirements predicted from the PLS model, the experimental result, and the percent prediction ability are also shown in Table 5.

3. Discussion

Identification test revealed that phenolics, flavonoids, and glycoside compounds are present in the extract. These results are in agreement with a previous study, which reported that the active compounds from *C. nutans* are vitexin, isovitexin, schaftoside, isomollupentin 7-O-bglucopyranoside, orientin, isoorientin, and quercetin, all of which are flavonoids (Wang and Zhao 2016). *C. nutans* also contain glycosides, glycoglycerolipids, cerebrosides, and monoacylmonogalatosylglycerol (Alam et al. 2016).

In this study, the effect and relation of three crucial formulation factors (PVP K30, glycerol, and acetone:ethanol ratio) on product properties were revealed and described by PLS modeling. The amounts of PVP K30 and glycerol in the formulation substantially intensified the stickiness of the spray solution. By contrast, the high ratio of acetone:ethanol combined with PVP K30 or glycerol reduced the stickiness. PVP K30 and glycerol act as viscosity inducing agent and humectant in the formulation, respectively (Nakashima et al. 2014). The bioadhesive property of both excipients can also enhance the formulation stickiness (Richardson et al. 2004). In addition, the presence of acetone in the formulation reduces the surface tension of the solution, thus resulting in low stickiness (Machado et al. 1999).

Viscosity can influence the spray performance; fluids with viscosity greater than 100 cPs are difficult to spray unless another additive or steam is provided (Center for Chemical Process Safety,

Table 5: Product requirement for optimization, evaluation results from predicted value from the PLS model and prediction ability

Response variable	Product requirement	Constraint (min – max)	Predicted value from PLS model	Experimental result	Prediction ability (%)
Stickiness (Y_1)	minimize	85.83 – 938.34 mN	92.24 mN	186.88 mN	49.35
Viscosity (Y_2)	minimize	3.17 – 35.90 cP	4.63 cP	3.83 cP	79.11
Evaporation rate (Y_3)	minimize	0.0584 – 0.1076%	0.0959%	0.0915%	95.19
Radius of spray coverage (Y_4)	maximize	6.26 – 9.60 cm	9.40 cm	13.63 cm	68.96

1996). In this study, the viscosity of all formulations was lower than 30 cPs, which is a suitable value for a liquid spray formulation. According to the PLS model, PVP K30 and glycerol played a vital role in the viscosity of the formulation. The increment in acetone proportion in the solvent system reduced the viscosity. No inter-factor effect was observed from this model. PVP K30 is a hydrophilic polymer that could increase the viscosity of a solution for spraying (Schroder et al. 2013). Glycerol also slightly enhanced the formulation viscosity. Glycerin has humectant and antimicrobial activities that can improve the properties of the topical formulation.

Fast evaporation can improve the ease of use of liquid spray formulations. In this study, PVP K30 primarily slowed the evaporation time, and the high proportion of acetone accelerated the evaporation. This phenomenon occurred because soluble polymer obscures the surface of the solution and prevents water molecules from evaporating (Wu et al. 2011). In addition, acetone has a low boiling point (56 °C), and increasing its portion in the formulation solvent can accelerate the evaporation rate (Chen 1983).

Acetone did not affect the size of the spray coverage. However, mixing acetone, PVP K30, and glycerol significantly reduced the spray area. The high proportion of PVP K30 and glycerol in the formula possibly increased the surface tension of the solution. Solutions with high surface tension form large spraying drops, which result in an increased solution drop weight that reduces the movement and distance of the spraying drop at a similar propellant spraying force (Christanti and Walker 2001).

During optimization, the PLS model successfully predicted viscosity (Y_2) and evaporation rate (Y_3) responses with prediction ability percentages of 79.11% and 95.19%, respectively. However, the model was unsuitable for predicting the stickiness (Y_1) and radius of spray coverage (Y_4). A possible reason is because the data factors and responses (Y_1 or Y_4) were unrelated or did not have a linear relationship; hence, the two-level full factorial design cannot describe or reveal these relations (Fang and Qin 2005).

In summary, the PLS model revealed that increasing the amount of PVP K30 and glycerin in the formulation also increases the stickiness and viscosity of the solution. By contrast, the use of high PVP K30 and glycerin concentrations can limit the spraying area and marginally decrease the evaporation rate. Increasing the acetone percentage in the solvent system reduces the formulation's stickiness and viscosity. Finally, the topical liquid spray formulation of *Clinacanthus nutans* was prepared and optimized. The obtained optimized formulation provided low viscosity (less than 3.83 cPs), moderate stickiness (186.88 mN), and wide spray coverage (13.63 cm) appropriate for liquid spay formulations. Therefore, the PLS model can predict the formulation properties of viscosity and evaporation rate. All these results indicated that the optimized topical liquid spray formulation is applicable for actual practice. In addition, the DOE quadratic model can be applied in future works to disclose formulation factors and properties.

4. Experimental

4.1. Materials

The leaf and leaf stalks of *C. nutans* were collected in Ratchaburi Province (Thailand). PVP K30 (lot no. 03900236009) was purchased from P.C. Drug Center Co., Ltd., Thailand. Glycerol (lot no. 000149) was obtained from Vidhyasom Co., Ltd. (Thailand), and PEG 400 (lot no. 1366280) was received from Pluka, Thailand. All other excipients were of pharmaceutical grade, and other chemicals were of reagent grade.

4.2. Extraction method

The leaf and leaf stalks of *C. nutans* were dried and comminuted by tray dryer and fritz mill, respectively. Extraction was carried out by macerating the 450 g of dried-milled product in 3 L of 95% ethanol for 14 days. The liquid extract was filtered and then partitioned by hexane for three times in separate funnels to eliminate chlorophyll that may interfere in the analysis of flavonoids. The extracted product was kept in a container under light protection and ambient temperature for further identification and spray solution preparation.

4.3. Compound identification and flavonoid quantification from the extracts

Three identification tests, namely, ferric chloride test, cyanidin test, and Molish's test were utilized to identify the presence of phenolics, flavonoids, and glycoside compounds in the extracts, respectively (Loganathan et al. 2017). Ferric chloride test was conducted by dropping ferric chloride solution on the extracts. The color altering of solution was monitored. A color change to dark brown indicates the presence of phenolic compound. In the cyanidin test, two pieces of magnesium filings (ribbon) were added to the herb extract. A concentrated hydrochloric acid solution was then dropwise added to the solution. Color change was observed to check the presence of flavonoids. In the last identification test (Molish's test), 5% α-naphthol solution in ethanol was dropwise added into the extract, followed by 2 mL of concentrated sulfuric acid to examine the glycoside structure.

The total flavonoid content in *C. nutans* extract was quantified using a previous method (Kalita et al. 2013). In brief, 1.5 mL of methanol, 0.1 mL of 10% aluminum chloride, and 0.1 mL of potassium acetate were added and mixed with *C. nutans* extract. The mixed solution was adjusted to 5 mL volume and then kept at ambient temperature for 30 min. The concentration of flavonoid was measured by T60 UV-vis spectrophotometer (PG instrument Ltd., Leicestershire, UK) at a wavelength of 415 nm. The measured absorbance value was compared with the reference standard of flavonoid (quercetin) to calculate the concentration of flavonoid in the *C. nutans* extract. The flavonoid quantification test was run in triplicate.

4.4. Formulation of topical liquid spray by experimental design

The amounts of active ingredient and other excipients of the topical liquid spray formula are shown in Table 6. Phosphate buffer was employed to modify the pH of the formulations to 5.1, which is close to the pH of the skin. Three critical factors of the formula, namely, the amounts of PVP K30 (X_1) of glycerol (X_2) and the ratio of acetone:ethanol (X_3) were chosen to reveal the effect viscosity inducing agent, humectant, and co-solvent on the product properties (Table 7). The experiment was designed using a two-level full factorial model. Eight experimental runs were performed in triplicates. The prepared topical spray solution was evaluated by four response variables, namely, stickiness (Y_1), viscosity (Y_2), evaporation rate (Y_3), and spray coverage radius (Y_4). After the designed experiments were completed, the PLS models were generated to find and predict the fundamental relations between two matrices (response and independent variables). The optimized formula was then engendered, prepared, and tested.

Table 6: Master formula of the *Clinacanthus nutans* extract-loaded topical spray solution

Ingredients	Master formula
Clinacanthus nutans extract	2 mL
Polyvinyl pyrrolidone K30*	0.1-10 g
Glycerol*	1-20 mL
Acetone:Ethanol (20:80-80:20)*	30 mL
PEG 400	5 mL
Sodium bisulfite	0.1 g
Monosodium phosphate	8.84 g
Dibasic sodium phosphate	0.32 g
Hydrochloric acid	qs
Sodium hydroxide	qs
Flavor	qs
Purified water	qs to 100 mL

Note: * indicates selected critical excipients (independent variables) for factorial design.

Table 7: Experimental design by 2-level full factorial model

Factor	Level	Code	
X_1	Polyvinyl pyrrolidone K30 (PVP K30)	0.1%	-
		10%	+
X_2	Glycerol	1%	-
		20%	+
X_3	Acetone : Ethanol	20:80	-
		80:20	+

Note: The amount, concentration or ratio used in the design of experiment was determined by the preliminary study.

4.5. Evaluation of stickiness

The stickiness level of the topical liquid spray was examined by texture analyzer (TA.XT plus, Stable Micro Systems, UK), and the test conditions are presented in Table 8. The instrument was calibrated prior to probe attachment. In brief, 0.2 mL of the topical spray solution was dropwise added on a flat surface aluminum plate. The detached force of probe from the plate was recorded. Each experiment was conducted in triplicate.

Table 8: Test conditions of the texture analyzer

Parameter	Value
Pre-test speed	1.0 mm/s
Test speed	0.5 mm/s
Post-test speed	0.5 mm/s
Applied force	0.05 N
Contact time	60 s
Probe selection	P/20P (20-mm diameter cylindrical probe)

4.6. Determination of viscosity

The viscosity of the spray solution was determined by a viscometer (DV II+ Pro model, Brookfield, USA) using spindle number CPE-40 at speed of 100 rpm at ambient temperature. The measurements were accomplished in triplicate, and the average of three readings was recorded.

4.7. Evaluation of evaporation rate

The evaporation rate of the spray solution was monitored by gravimetric technique. In brief, 0.2 mL of spray solution was dropwise added on 1 square inch filter paper. Its weight at initial time and after 5 min was recorded by a four-digit analytical balance. The percent weight change was calculated and compared (Huanbutta and Suttikijyothin 2017).

4.8. Radius of spray coverage

Different solution viscosities and surface tensions could provide different areas of spray coverage. Therefore, spray coverage efficiency was evaluated in all experimental design runs. Food additive color (45.5% sunset yellow and 6.5% Ponceau 4R) was added to the formulation to trail the spraying size. The solution was sprayed on a paper from fixed distance. After the paper had dried, the radius of spraying zone was measured and compared. Each experiment was performed in triplicate.

4.9. Statistical analysis

ANOVA and Levene's test for homogeneity of variance were performed using Minitab version 19 for Windows (SPSS Inc., USA). Post hoc testing ($p < 0.05$) for multiple comparisons was conducted using Scheffé or Games–Howell test depending on whether Levene's test was insignificant or significant, respectively (Sriamornsak et al. 2010).

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