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Optimization of complex coacervation by central composite design (CCD) : microcapsules of vitamin U using multiple emulsion method

중심계획법을 이용한 복합 코아세르베이션의 최적화 : 다중유화법을 이용한 비타민 U의 마이크로캡슐

2017년 8월

서울대학교 대학원
약학과 약제과학 전공
김 지 수
중심계획법을 이용한
복합 코아세르베이션의 최적화 : 다중유화법을 이용한 비타민 U의
마이크로캡슐
지도교수 김 대 덕

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서울대학교 대학원
약학과 약제과학 전공
김 지 수

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위원장 김 성자 (문
부위원장 이 우식 (인)
위원 김 대 덕 (인)

서울대학교
SEOU NATIONAL UNIVERSITY
ABSTRACT

Optimization of complex coacervation by central composite design (CCD) : microcapsules of vitamin U using multiple emulsion method

Ji-Su Kim
Dept. of Pharmaceutics, College of Pharmacy
The Graduate School
Seoul National University

Purpose
The purpose of this study was to prepare microcapsules encapsulating hydrophilic vitamin U (VU, methylmethionine sulfate chloride, MMSC) using complex coacervation. The composition of these particles was optimized using central composite design (CCD), which is one of response surface methodology (RSM), and characterized.
Methods

Since the complex coacervation reaction is known as a useful method for encapsulating hydrophobic materials, multiple emulsion method and s/o dispersion method were introduced for encapsulating highly water-soluble vitamin U (clogP = -1.7072, Chemdraw®, cambridgesoft). A ratio of gelatin and gum arabic which are components in particle (X1, weight of gelatin / weight of gum arabic) and a volume of oil phase (X2) were set as two variables to optimize acquired weight of microcapsules (Y1) to maximize and to obtain a VU content of more than 2.5% in microcapsule (Y2). Microcapsules prepared under optimal conditions were analyzed for identifying their properties using an optical microscope, a fluorescence microscope, a scanning electron microscope, a DSC and a laser diffraction particle size analyzer. Odor of VU was quantitatively identified using a headspace-gas chromatograph / mass spectrometer (HS-GC / MS).

Results & Discussion

When the microcapsules were prepared by applying the s/o dispersion method, a VU content was very low (0.001 ± 0.0003%). Thus, the composition of microcapsules using multiple emulsion method was optimized. When the microcapsules were actually prepared under optimal conditions, the predicted Y1 and Y2 values corresponded to their actual measured values over 95%. Therefore, it can be concluded that this model was well designed.

The morphology of microcapsules encapsulating multiple emulsion was identified by using the optical microscope and the fluorescence microscope. In addition, the SEM image of internal particle also indicated that the microcapsule encapsulated
multiple emulsion. However, as a result of the analysis using a laser diffraction particle size analyzer, the size was not uniform (average particle size 79.17 ± 69.82 μm) and showed a wide particle size distribution. The DSC results showed that the materials that constitute the particles (vitamin U, transglutaminase, and gelatin) exist in an amorphous state in the coacervate microcapsules. The amount of dimethylsulfide, which is the cause of vitamin U odor, was analyzed by HS-GC/MS. As a result, the amount of dimethysulfide detected in microcapsules containing 6.25 mg of vitamin U was DMS generated from about 70 mg of vitamin U powder. This suggests that the microcapsule manufacturing process affected the stability of vitamin U, and further studies are needed to improve it.

Conclusion

The hydrophilic vitamin U was successfully encapsulated in the coacervate microcapsules using the multiple emulsion method, and the DoE technique was found to be a useful method for finding the optimum condition. It is believed that the formulation is applicable to cosmetics and pharmaceuticals in the future.

**keywords:** Complex coacervation, Microcapsule, Vitamin U, Multiple emulsion, Response surface methodology, Central composite design

**Student Number : 2015-23179**
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1. Introduction

Complex coacervation is a phenomenon that occurs in two polymers under acidic conditions generally. Under acidic conditions, one polymer is positively charged, and the other polymer is negatively charged. Two polymers then coagulate electrostatically, followed by occurring phase separation and coacervate microcapsules are formed with precipitation (Comunian et al., 2013; Rocha-Selmi et al., 2013; Santos et al., 2014; van der Burgh et al., 2004). Combinations of two commonly used polymers include gum arabic/gelatin, gelatin/carboxymethylcellulose, albumin/gum arabic and alginate/polylysine (Hong and McClements, 2007; Oliveira et al., 2007; Qv et al., 2011).

Complex coacervation is a promising preparation method which can be used in many industries, including food, cosmetics and pharmaceutics. It enables to encapsulate toxic materials, promote controlled release and stabilize products, isolate products, and even mask unpleasant odors or unflavorable taste (Comunian et al., 2013; Qv et al., 2011).

Vitamin U (VU, methylmethionine sulfonium chloride, MMSC, S-methylmethionine), which is known to be effective against various gastrointestinal diseases, was reported to have various advantages in skin. First of all, when applied to the skin, it has anti-wound and photoprotective effect by protecting keratinocyte progenitor cells (KPCs) and human dermal fibroblast (hDF) from ultraviolet B irradiation (Kim et al., 2015). VU is also known to have anti-inflammatory effect (Kim et al., 2010; Urazaeva, 1976). Therefore, it can be used as a potent cosmetic raw material.
VU is a hydrophilic material. The clogP value of VU calculated by Chemdraw® (Cambridgesoft, MA, USA) was -1.7027. Since the complex coacervation reaction is a useful preparation method for encapsulating hydrophobic materials (i.e. oils) application of a complex coacervation to VU required a special method. In this study, multiple emulsion method and s/o dispersion method were introduced into complex coacervation for encapsulating VU (Comunian et al., 2013; Rocha-Selmi et al., 2013; Santos et al., 2014).

Quality by design (QbD) methodology has been used widely in many fields including the pharmaceutical industry. The concept of QbD can improve quality management and understanding of manufacturing processes and products (Rathore and Winkle, 2009). It can be a solution that can support the industry in a progressive and scientific approach. Response surface methodology (RSM) is one of the statistical QbD methodologies, and is often used to optimize the response among several variables (Baş and Boyacı, 2007). RSM is a combination of statistical and mathematical techniques, which is useful for developing and optimizing processes as well as analyzing the relationship between independent variables and response variables. (Anjum et al., 1997; Baş and Boyacı, 2007). There are several advantages of process optimization by RSM. First of all, unlike classical methods, RSM is time-efficient and can provide a lot of information in a few experiments. Second, it is useful to observe the interaction of the independent parameters using RSM. Thus, RSM is a convenient tool for optimization process. However, there is a drawback to use RSM. It is possible to fit data to only a second-order polynomial. Some systems, such as Michaelis-Menten equation curvature, can not be accommodated by second order polynomial (Anjum et al.,
The purpose of this study was to prepare microcapsules encapsulating hydrophilic VU using complex coacervation. The composition of microcapsules was optimized using central composite design (CCD), which is one type of RSM, and characterized particles including morphology and particle size.
2. Material and methods

2.1 Material

DL-Methionine methylsulfonium chloride (MMSC, vitamin U, VU) and olive oil were purchased from Sigma-aldrich Co. (St. Louis, MO, USA). Gelatin from cold water fish skin and gum arabic from acacia tree for the wall component of microcapsules were also purchased from Sigma-aldrich Co. (St. Louis, MO, USA). TWEEN80 and diethylene glycol monoethyl ether (Transcutol®) used as surfactant for preparing the multiple emulsion were also purchased from Sigma-aldrich Co. (St. Louis, MO, USA). A transglutaminase (103U/g) used for crosslinking reaction was purchased from Ajinomoto (Activa TG-S®, Tokyo, Japan).

2.2 Preparation of coacervate microcapsules encapsulating w/o/w emulsion of VU

A method of complex coacervation encapsulating VU was established according to previously published articles about coacervate microcapsules encapsulating hydrophilic materials (Devi et al., 2012; Dong et al., 2008; Jun-xia et al., 2011; Mendanha et al., 2009; Nori et al., 2011; Santos et al., 2014) with some revisions. To produce a primary emulsion (W/O), 0.3ml of VU solution (which can be prepared with 2500mg of VU dissolved in 1ml DI water) was added to 0.3ml of oil phase (olive oil with 10% (v/v) Transcutol®) and homogenized (T25 model, IKA, Staufen, Germany) for 3 min at 12,000 rpm. To produce a multiple emulsion (w/o/w), the
primary emulsion was gently added to external aqueous phase solution in which gelatin and gum arabic (total 1.25 g) were dissolved in 25 ml DI water in presence of 2% (v/v) Tween80. They were homogenized for 30 sec at 8000 rpm. Immediately after w/o/w emulsion was prepared, 10% acetic acid was added to adjust pH below 4.0 under constant magnetic stirring at 500 rpm for complex coacervation reaction. The complex coacervation reaction proceeded for 1 hr. After the coacervation reaction, transglutaminase (10 U/g gelatin) was added to the solution as a crosslinking agent. The crosslinking reaction was proceeded for at least 12 hr. After that, the product was washed with a washing solution, which was prepared with DI water adjusted pH below 4 using 10% acetic acid, to remove the remaining VU and other components. About 50 ml of washing solution was added to dilute supernatant. It was left without any mixing for 30 min to make the microcapsules move beneath. After phase separation into coacervate phase and upper phase, the upper phase was removed. Subsequently, the remaining particles were dried using a freeze-dryer, FDCF-12003 (Operon, Gimpo, Korea), to block aggregation between the microcapsules and to facilitate storage of microcapsule. The microcapsules were freeze-dried at -70 °C for 48 hr. These all procedures are shown in Figure 1.

2.3. Preparation of coacervate microcapsules encapsulating s/o dispersion of VU

100 mg of VU powder was placed on the bottom of a 2.0 ml EP tube and the oil phase (1.5 ml) used for multiple emulsion method was added. A probe-sonicator, model VC750 (Sonics & Mateirlas Inc., CT, USA) was used for 1 min at 25
amplitude to disperse VU into the oil phase. The supernatant (1ml) after 1 hr standing without any mixing (s/o dispersion), was used to prepare complex coacervation in the same way as used for multiple emulsion method. Three batches were prepared to analyze VU content in microcapsules. These all procedures are shown in Figure 2.

2.4 Response surface methodology (RSM)

The VU-loaded coacervate microcapsules encapsulating w/o/w emulsion were optimized using statistical software Minitab 17® for RSM modeling. Central composite design (CCD) is a model of RSM, which is used to optimize variables more than two (Hibbert, 2012). Throughout this study, the central composite design (CCD) was used to optimize the formulation with the maximum acquired weight of microcapsules (Y1) and VU content of 2.5% or more in microcapsules (Y2). Independent variables used in this study were wall ratio (gelatin/gum arabic, total weight of wall components was 1250 mg, X1) and amount of oil phase (ml, X2). The independent variables (X1 and X2) and the response variable (Y1 and Y2), theirs goal, importance and weight used in this study are listed in Table 1. The fourteen experiments were designed by CCD and the response variables obtained by actual measurement are shown in Table 2. The combination of independent variables was built from designated as axial point and set 2 block scheme.

2.5 Characterization of the coacervate microcapsules

2.5.1 Morphological characterizations of the microcapsules : optical
The morphology of the microcapsules was characterized by an optical & fluorescent microscopes (Olympus, model IX70, Japan) equipped with a ‘analySIS TS Lite’ program. The wet microcapsules (prior to freeze-drying) were placed on a slide glass to fix for observation. The lipophilic fluorescent dye, 100μl of DiI in DMSO (0.1ug/ml) dissolved in oil phase was used for the fluorescent microscope image.

A surface of freeze-dried microcapsules was characterized by scanning electron microscope (Hitachi TM3000, Tokyo, Japan). Freeze-dried microcapsules were adhered to the carbon tape. The sample was no pre-treated for SEM image. An accelerating voltages of 15 kV was used to observe the surface of sample.

2.5.2 Differential scanning calorimetry (DSC)

Thermal characteristic of the freeze-dried microcapsules, together with its ingredients (gelatin, gum arabic, oil phase, VU and transglutaminase) was determined by differential scanning calorimetry using DSC-Q1000 model (TA Instrument, New Castle, DE, USA). Each sample was heated from 25 °C to 200 °C at a rate of 10 °C per minute.

2.5.3 Size analysis

The size of microcapsules and the distribution of size were determined by the laser light scattering method. The Microtrac S3500 (Nikkiso, Tokyo, Japan) was used,
which can measure sizes ranging from 0.02 to 2800 μm. The freeze-dried microcapsules were dispersed in DI water for size analysis.

2.5.4 *VU content measurement by LC-MS/MS*

The LC-MS/MS was used for analysis of VU content by previous article (Kim et al., 2017). The freeze-dried microcapsules were dispersed in DI water to a concentration of 10 mg/ml. And the dispersed liquid was centrifuged under the following conditions to break the microcapsules for dissolving the encapsulated VU into DI water: 6 min, 40 °C, 13200 rpm. And then it was analyzed by LC/MS/MS after dilution with standard samples for calibration curve. Details of condition are follows: The used LC-MS/MS system was combined Agilent Technologies 1260 Infinity HPLC system (Agilent Technologies, SantaClara, CA, USA) and Agilent Technologies 6430 TripleQuad LC/MS system (Agilent Technologies, SantaClara, CA, USA). Gas temperature, gas flow, nebulizer pressure and capillary voltage in this system was set to 300 °C, 11 L/min, 15 psi, 4500 V (+) charge mode. The column used for separating VU from other components was C12 column, Synergi Max-RP 5μ (Phenomenex, California, CA, USA) and injection volume was 3 μL. Mobile phase used for analysis consisted of acetonitrile and distilled water (73:27, v/v) in presence of 0.2% formic acid and flow rate was 0.5 mL/min. The m/z value of precursor/product ion of VU was 163.9 and 102.0, respectively and fragment voltage was 73V. In this conditions, retention time of VU was 1.04 min. The lower limit of quantification (LLOQ) of VU was 20 ng/ml.
2.5.5 Odor masking evaluation

A previous article reported that VU can be easily degraded into dimethylsulfide (DMS) during storage (Akpolat and Barringer, 2015). Therefore, DMS can be used as an indicator of VU odor. Aliquot (250 mg) of optimal microcapsules were used for HS-GC/MS analysis. DMS measurement were performed in HS-GC/MS system (HS : TurboMatrix40, Perkinelmer, Massachusetts, MA, USA; GC : Clarus 680, Perkinelmer, Massachusetts, MA, USA; MS : Clarus 600T, Perkinelmer, Massachusetts, MA, USA). Details of condition are followings: In the GC system, initial temperature of oven was set to 35 °C for 2 min, and ramped 5 °C/min to 45 °C, hold 8 min and ramped 50 °C/min to 200 °C, hold 1 min. Temperature of injector was 250 °C. And carrier gas was He at 15 psi. Oven temperature and heating time in the headspace conditions were set to 35 °C and 20 min, respectively. In the MS system, solvent delay, transfer temperature and source temperature were 3.5 min, 250 °C and 250 °C respectively. Scan ranges was from 35 to 250 Da.
3. Result

3.1 Preliminary study: content of vitamin U in microcapsules using s/o dispersion method and multiple emulsion method

The content of vitamin U in the microcapsules using s/o dispersion method was analyzed to 0.001±0.0003% whereas VU content of microcapsules using multiple emulsion method was over 1%. Therefore, the s/o dispersion method was not an adequate approach for complex coacervation of VU.

3.2 Optimization by central composite design and 3D-response surface plots

The central composite design (CCD) was employed to optimize the microcapsules. The response surface regression results are shown Table 3. They showed that Y1 and Y2 were significantly influenced by X1 (p < 0.01) while Y1 was significantly influenced by only X2 (p < 0.01). And Y1, Y2 can be explained with the following second order polynomial equations:

\[
Y_1 = -212 + 1633 \times X_1 + 110 \times X_2 - 867 \times X_1 \times X_1 - 63.2 \times X_2 \times X_2 + 121 \times X_1 \times X_2
\]
\[
Y_2 = 4.907 - 3.84 \times X_1 - 0.572 \times X_2 + 1.374 \times X_1 \times X_1 - 0.0608 \times X_2 \times X_2 + 0.735 \times X_1 \times X_2
\]

And this model have no significant lack of fit value.

The surface plots and contour plots (Figure 3-a ~ d) were used to find optimal point for X1, X2. The countour plots of Y1, Y2 versus X1, X2 are shown Figure 3-a,
Figure 3-b, respectively. And the surface plots of Y1, Y2 versus X1, X2 are shown Figure 3-c, Figure 3-d, respectively. As a result of the optimization, the optimal X1 was 1.0 and X2 was 1.9. The predicted values of Y1, Y2 when microcapsules were prepared under optimal conditions were 765.9 and 2.5, respectively according to the optimization plot (Figure 4).

Additionally, the R-squared values of each model (Y1 versus X1, X2 and Y2 versus X1, X2) were 0.9015 and 0.8976, respectively, which are closed to 1.0000. A comparison between the predicted values (Y1 and Y2) and the actual measurement values obtained by using optimized dependent variables is listed Table 4. Three batches of microcapsules under optimal conditions were prepared for anlaysis. The actual measurement values, Y1 and Y2, were analyzed to 748.3±56.3 (mg), 2.5±0.0 (%), respectively. The relative percentages (100 × measurement value / predicted value) of Y1, Y2 were 97.7% and 98.8%, respectively.

3.3 Morphological characteristics of the coacervate microcapsules by an optical microscopy, a fluorescent microscopy and a scanning electron microscopy (SEM)

The optical and fluorescent microscopy images (Figure 5-a, 5-b) indicated that the wet microcapsules exhibited circular shape and varied sizes, and encapsulating multiple emulsion without phase separation.

The surface of freeze-dried microcapsule was observed by SEM. After the pre-treatment process, the particles were burst and thus they did not a pre-treated. The
morphology of the inner microcapsules is shown in Figure 5-c. The many internal pores in the particle indicated that the microcapsule encapsulated multiple emulsion.

### 3.4 DSC

The DSC thermograms of VU, gelatin, gum arabic, transglutaminase, oil phase and coacervate microcapsules are shown in Figure 6. The coacervate microcapsules exhibited different thermal behavior compared to the components. The coacervate microcapsule did not show a clear peak of thermal behavior, while VU, gelatin and transglutaminase exhibited distinct endothermic peak at about 139 °C, 71 °C, 148 °C, respectively.

### 3.5 Size analysis

The microcapsules showed wide size distribution (Figure 7). The mean diameter of the volume distribution was measured to be 79.17±69.82 μm. The median diameter was measured to be 72.85 μm. Around 20% of the particles had a diameter over 137.2 μm and 80% of the particles had a diameter over 10.43 μm.

### 3.6 Odor evaluation

The volitile dimethylsulfide (DMS) concentration in the coacervate microcapsules encapsulating 6.25mg of VU was analyzed to have the same amount of DMS
generated from 69.9±17.7 mg of vitamin U powder (Figure 8).
4. Discussion

The microcapsules prepared by w/o/w emulsion method had about 2500-fold higher VU content compared with that by s/o dispersion method. Since, the complex coacervation using multiple emulsion method was more adequate for VU-loaded microcapsules. The microcapsules using multiple emulsion method were selected for optimizing.

The independent variables (X1 and X2) were optimized by CCD modeling. The response surface regression results indicated that this model describes Y1, Y2 well, because this model have no significant lack of fit value (Sanchez et al., 2016) and the R² value of each model was close to 1.0000. The relative percentages of actual measurement value versus predicted value were all more than 95%. It indicated that the regression models were well predicted. Therefore, it can be concluded that this model was well-established for optimization of VU-loaded microcapsules.

The morphology of wet microcapsules (prior to freeze-drying) with optical and fluorescent microscope indicates that the coacervate microcapsules can encapsulate multiple emulsion without phase separation. This morphology differed from the particles obtained by Guo (Guo and Zhao, 2008) whose microcapsules did not encapsulate multiple emulsion. The oil phase (containing DiI, the lipophilic fluorescent dye) encapsulated in the microcapsule was identified from red signal in the fluorescence microscope image. A internal morphology of the freeze-dried microcapsules by the SEM image revealed the multiple emulsion encapsulated in microcapsules from many pores in the inner particle. This morphology was similar with particles obtained by Alvim (Alvim and Grosso, 2010) whose microcapsules
encapsulated multiple emulsion.

As expected from the microscope images, the size distribution measured by laser light scattering particle size analyzer was very broad. This tendency was consistent with previous articles reporting as from 0.96 μm to 680 μm (Builders et al., 2008; Kong et al., 2009; Prata et al., 2008).

The endothermic peaks of gelatin, transglutaminase and VU did not appear in the thermogram of coacervate microcapsules. This result indicates that these components remained in amorphous form and decomposited state in the coacervate microcapsules.

From HS-GC/MS result, the DMS concentration of VU in coacervate microcapsules (containing 6.25 mg of VU) was analyzed to DMS generated from about 10-folded vitamin U powder. Therefore, it can be concluded the odor of VU cannot be masked by complex coacervation.
5. Conclusion

The central composite design successfully optimized complex coacervation of hydrophilic vitamin U using multiple emulsion method. The predicted values of the two response variables were well fitted. It was confirmed that the particles encapsulate hydrophilic VU in multiple emulsion form. They had spherical shape with their sizes ranging from less than 1 μm to more than 200 μm. Thus, this coacervate could be further developed in cosmetics, food and medicine industries.
6. Reference


Table 1. Levels of Independent variables (X1 and X2) and goal, importance and weight of response variables (Y1 and Y2).

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<thead>
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<th>Independent variables</th>
<th>Levels</th>
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<td>X1 : Wall ratio</td>
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<td>X2 : Amount of oil phase (ml)</td>
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<td>Y2 : VU Content (%)</td>
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Table 2. Experimental combination of independent variables by central composite design and response results.

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<td>Amount of oil phase (ml)</td>
<td>Acquired weight (mg)</td>
<td>VU content (%)</td>
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</tr>
<tr>
<td>8</td>
<td>0.900</td>
<td>1.050</td>
<td>738</td>
<td>2.77</td>
</tr>
<tr>
<td>9</td>
<td>0.900</td>
<td>1.050</td>
<td>705</td>
<td>2.62</td>
</tr>
<tr>
<td>10</td>
<td>0.900</td>
<td>2.000</td>
<td>740</td>
<td>2.60</td>
</tr>
<tr>
<td>11</td>
<td>1.200</td>
<td>1.050</td>
<td>710</td>
<td>2.58</td>
</tr>
<tr>
<td>12</td>
<td>0.900</td>
<td>1.050</td>
<td>672</td>
<td>2.82</td>
</tr>
<tr>
<td>13</td>
<td>0.900</td>
<td>0.100</td>
<td>568</td>
<td>2.64</td>
</tr>
<tr>
<td>14</td>
<td>0.600</td>
<td>1.050</td>
<td>556</td>
<td>3.02</td>
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</table>
Table 3. Response surface regression: Acquired weight (Y1) versus wall ratio (X1), amount of oil phase (X2) and VU content (Y2) versus X1 and X2.

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
<th>Contribution</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Response surface regression of Y1 versus X1, X2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>59056.6</td>
<td>90.15%</td>
<td>9842.8</td>
<td>10.68</td>
<td>0.003**</td>
<td></td>
</tr>
<tr>
<td>Blocks</td>
<td>1</td>
<td>728.6</td>
<td>1.11%</td>
<td>728.6</td>
<td>0.79</td>
<td>0.403</td>
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</tr>
<tr>
<td>Linear</td>
<td>2</td>
<td>41056.0</td>
<td>62.67%</td>
<td>20528.0</td>
<td>22.27</td>
<td>0.001**</td>
<td></td>
</tr>
<tr>
<td>X1</td>
<td>1</td>
<td>14347.2</td>
<td>21.90%</td>
<td>14347.2</td>
<td>15.57</td>
<td>0.006**</td>
<td></td>
</tr>
<tr>
<td>X2</td>
<td>1</td>
<td>26708.8</td>
<td>40.77%</td>
<td>26708.8</td>
<td>28.98</td>
<td>0.001**</td>
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</tr>
<tr>
<td>Square</td>
<td>2</td>
<td>16081.7</td>
<td>24.55%</td>
<td>8040.9</td>
<td>8.72</td>
<td>0.013b)</td>
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</tr>
<tr>
<td>X1*X1</td>
<td>1</td>
<td>10074.8</td>
<td>15.38%</td>
<td>11244.0</td>
<td>12.20</td>
<td>0.010</td>
<td></td>
</tr>
<tr>
<td>X2*X2</td>
<td>1</td>
<td>6006.9</td>
<td>9.17%</td>
<td>6006.9</td>
<td>6.52</td>
<td>0.038a)</td>
<td></td>
</tr>
<tr>
<td>2-Way Interaction</td>
<td>1</td>
<td>1190.2</td>
<td>1.82%</td>
<td>1190.2</td>
<td>1.29</td>
<td>0.293</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>7</td>
<td>6452.3</td>
<td>9.85%</td>
<td>921.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack-of-Fit</td>
<td>3</td>
<td>1369.6</td>
<td>2.09%</td>
<td>456.5</td>
<td>0.36</td>
<td>0.787</td>
<td></td>
</tr>
<tr>
<td>Pure Error</td>
<td>4</td>
<td>5082.7</td>
<td>7.76%</td>
<td>1270.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>65508.9</td>
<td>100.00%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Seq SS</th>
<th>Contribution</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Response surface regression of Y2 versus X1, X2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Model</td>
<td>6</td>
<td>0.391902</td>
<td>89.76%</td>
<td>0.065317</td>
<td>10.22</td>
<td>0.004**</td>
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<td>Blocks</td>
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<td>0.178766</td>
<td>40.94%</td>
<td>0.178766</td>
<td>27.98</td>
<td>0.001**</td>
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<tr>
<td>Linear</td>
<td>2</td>
<td>0.133323</td>
<td>30.53%</td>
<td>0.066661</td>
<td>10.43</td>
<td>0.008**</td>
<td></td>
</tr>
<tr>
<td>X1</td>
<td>1</td>
<td>0.128100</td>
<td>29.34%</td>
<td>0.128100</td>
<td>20.05</td>
<td>0.003**</td>
<td></td>
</tr>
<tr>
<td>X2</td>
<td>1</td>
<td>0.005222</td>
<td>1.20%</td>
<td>0.005222</td>
<td>0.82</td>
<td>0.396</td>
<td></td>
</tr>
<tr>
<td>Square</td>
<td>2</td>
<td>0.035923</td>
<td>8.23%</td>
<td>0.017962</td>
<td>2.81</td>
<td>0.127</td>
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</tr>
<tr>
<td>X1*X1</td>
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<td>0.030373</td>
<td>6.96%</td>
<td>0.028234</td>
<td>4.42</td>
<td>0.074</td>
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</tr>
<tr>
<td>X2*X2</td>
<td>1</td>
<td>0.005551</td>
<td>1.27%</td>
<td>0.005551</td>
<td>0.87</td>
<td>0.382</td>
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</tr>
<tr>
<td>2-Way Interaction</td>
<td>1</td>
<td>0.043890</td>
<td>10.05%</td>
<td>0.043890</td>
<td>6.87</td>
<td>0.034a)</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>7</td>
<td>0.044728</td>
<td>10.24%</td>
<td>0.006390</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lack-of-Fit</td>
<td>3</td>
<td>0.020638</td>
<td>4.73%</td>
<td>0.006879</td>
<td>1.14</td>
<td>0.433</td>
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</tr>
<tr>
<td>Pure Error</td>
<td>4</td>
<td>0.024089</td>
<td>5.52%</td>
<td>0.006022</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Total</td>
<td>13</td>
<td>0.436630</td>
<td>100.00%</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

DF : degrees of freedom
Seq SS : sequential sum of squares
Adj SS : adjusted sum of squares
Adj MS : adjusted mean squares

a) shows a highly significance, p<0.01
b) shows a significance, p<0.05
Table 4. Comparison between predicted Y1 and Y2 obtained from CCD and actual measurement Y1 and Y2 prepared under optimal conditions.

<table>
<thead>
<tr>
<th></th>
<th>Predicted value (A)</th>
<th>Actual measurement value (B)</th>
<th>Relative percentage (100 × B/A)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y1</td>
<td>765.9 (mg)</td>
<td>748.3±56.3 (mg)</td>
<td>97.7 (%)</td>
</tr>
<tr>
<td>Y2</td>
<td>2.5 (%)</td>
<td>2.5±0.0 (%)</td>
<td>98.8 (%)</td>
</tr>
</tbody>
</table>

Y1 : Acquired weight, mg  
Y2 : VU content, %
Figure 1. Schematic illustration of the preparation of complex coacervate microcapsules of VU using multiple emulsion method.
Figure 2. Schematic illustration of the preparation of complex coacervate microcapsules of VU using s/o dispersion method.
Figure 3. a) Contour Plot of acquired weight (Y1) versus wall ratio (X1), amount of oil phase (X2). b) Surface Plot of Y1 versus X1 and X2. c) Contour Plot of VU content (Y2) versus X1 and X2. d) Surface Plot of Y2 versus X1 and X2.
<table>
<thead>
<tr>
<th>X1</th>
<th>High</th>
<th>Cur</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.20</td>
<td>1.0121</td>
<td>0.60</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>X2</th>
<th>High</th>
<th>Cur</th>
<th>Low</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.0</td>
<td>1.9232</td>
<td>0.10</td>
<td></td>
</tr>
</tbody>
</table>

**Optimal Predict**

| Composite Desirability D: 0.9786 |

**Y1 Maximum**
y = 765.9166
d = 0.99921

**Y2 Target: 2.50**
y = 2.5322
d = 0.93852

X1 : Wall ratio, gelatin/gum arabic, total 1.25 g
X2 : Amount of oil phase, ml
Y1 : Acquired weight, mg
Y2 : VU content, %

Figure 4. Optimization plots of Y1 and Y2 versus X1 and X2.
Figure 5. a) Optical microscopic image of VU-loaded microcapsules (prior to freeze-drying) encapsulating multiple emulsion. The scale bar is 50 μm. b) Fluorescence microscopic image of VU-loaded microcapsules (prior to freeze-drying) encapsulating multiple emulsion. The scale bar is 50 μm. Red fluorescence signal is DiI, lipophilic fluorescent dye, dissolved in oil phase. c) Micrograph of internal freeze-dried VU-loaded microcapsules by scanning electron microscopy. The scale bar is 20 μm.
Figure 6. DSC thermograms of ingredients and VU-loaded coacervate microcapsules.
Figure 7. Size distribution of VU-loaded microcapsules prepared under the optimal conditions.
Figure 8. Calibration curve of dimethylsulfide generated from vitamin U powder for quantification.
국문 초록

Purpose

본 연구의 목적은 수용성인 비타민 U (VU, methylmethionine sulfate chloride, MMSC)를 봉입한 마이크로캡슐 입자를 복합 코아세르베이션 (complex coacervation) 반응을 통해 제조하고, 이 입자의 조성을 반응 표면법 (Response surface methodology, RSM)의 한 종류인 중심계획법 (Central composite design, CCD)을 이용하여 최적화하고 입자의 특성을 평가하는 것이다.

Methods

복합 코아세르베이션 반응은 지류성 물질을 봉입하기에 적합한 방법으로 알려져 있으므로, 수용성이 매우 강한 비타민 U (clogP=-1.7072, Chemdraw®, cambridgesoft)를 봉입하기 위해서 다중유화법 (multiple emulsion method)과 solid-in-oil (s/o) 분산법을 응용하여 마이크로캡슐 입자를 제조하였다. 입자의 구성물질인 젤라틴 (gelatin)과 아라비아 검 (gum arabic)의 비율 (X1, 젤라틴의 중량/아라비아검의 중량)과 유 상을 구성하는 물질인 10% transcuto이 용해된 올리브유의 부피, 즉 오일상의 부피 (X2)를 두 개의 변수로 설정하고, 중심계획법을 통해서 얻어지는 입자의 총량 (Y1)을 최대화시키고, 제제 내의 비타민 U의 함량 (Y2)이 2.5% 이상 되도록 조성을 최적화 하였다. 이렇게 만들어진 마이크로캡슐 입자는 광학현미경 및 형광현미경, 주사 전자현미경, DSC, 레이저 회절 입도분석기를 통해 입자의 성상 및 성질을 확인하였고, 헤드스페이스-가스크로마트그래피/절량분석기 (HS-GC/MS)로 비타민 U의 냄새 차폐 여부를 정량적으로 확인하였다.
Results & Discussion

수용성인 비타민 U를 봉입하기 위해 s/o 분산법을 응용하여 마이크로캡슐을 제조한 경우에는, 비타민 U 함량이 매우 낮았으므로 (0.001±0.0003%), 다중유화법을 이용한 코아세르베이션 방법으로 조성을 최적화하였다. CCD 모델을 통해 Y1과 Y2가 최적화되는 변수들의 조합을 얻었고, 실제로 이 조합을 가지고 마이크로캡슐을 제조하였을 때, 예측되는 Y1값과 Y2값이 그것들의 실측값과 95% 이상 일치하였다. 이를 통한 결과, 이 모델이 잘 설계되었고, 최적의 조성을 찾는데 유용한 방법임을 알 수 있었다. 광학 현미경과 현미경을 사용해 입자의 형태를 관찰한 결과, 젤라틴 마이크로캡슐 내에 multiple emulsion을 봉입하고 있음을 확인하였다. 그러나, 레이저 회절 입도분석기를 통해 분석한 결과, 입자의 크기는 균일하지 않았고 (평균입자경 79.17±69.82 μm), 넓은 입도 분포를 보였다. 또한, SEM 이미지를 통해 입자 내부에 있는 많은 pore들을 확인하였으며, 이로서 multiple emulsion 형태가 입자 안에 봉입되어 있음을 알 수 있었다. DSC 결과를 통해서 입자를 구성하는 물질(비타민 U, transglutaminase, gelatin)들이 코아세르베이션 입자 안에서의 무정형의 상태로 존재한다는 것을 확인할 수 있었다. 비타민 U 냄새의 주원인인 디메칠실리드의 양을 HS-GC/MS로 분석한 결과, 6.25mg의 비타민 U를 함유하고 있는 마이크로캡슐에서 검출된 디메칠실리드의 양은 약 70mg의 비타민 U 파우더에서 발생하는 것과 일치하였다. 이는 마이크로캡슐의 제조 과정이 비타민 U의 안정성에 영향을 미쳤기 때문인 것으로 생각되며, 향후 이를 개선하기 위한 연구가 필요할 것으로 생각된다.

Conclusion

다중유화법을 이용하여 코아세르베이션 입자 내에 수용성인 비타민 U
를 성공적으로 봉입하였고, DoE 기법이 최적의 조성을 찾는 유용한 방법임을 알 수 있었다. 향후, 화장품과 의약품에 적용가능한 제형인 것으로 생각된다.

주요어 : 복합 코아세르베이션, 마이크로캡슐, 비타민 U, Multiple emulsion, 반응표면법, 중심계획법

학번 : 2015 – 23179