# OPTIMISATION OF PROCESSING CONDITIONS OF PECTIN/RED CLOVER EXTRACT NANOEMULSIONS FOR MEDICAL TEXTILE APPLICATIONS[1]

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***ABSTRACT:*** *Herein we report the generation of pectin(P)/red clover extract (RCE) nanoemulsions (NEs). To that end, P/RCE NEs were prepared employing ultrasonication process (0, 1, 5, and 10 min) both in the absence and presence of a surfactant mixture consisting of 1/3 Span® 80/Tween® 80. Droplet size and Zeta potential data were statistically evaluated. The most significant factor for RCE NEs was determined to be the presence of surfactant mixture. Minimum droplet size (prediction: 51±1 nm) could be achieved by determining ultrasonication time as 5 min by using 1/3 Span® 80/Tween® 80 for aqueous RCE NEs. Addition of the RCE NEs to pectin solutions increased the droplet sizes to a large extent (372-728 nm). The prepared P/RCE NEs could be used to coat any kind of fibrous structure to produce medical textiles for hormonal regulation.*

***Keywords:*** *Red clover extract, pectin, surfactant, ultrasonication, nano emulsion.*

# OPTIMIZACIJA USLOVA PRERADE NANOEMULZIJA PEKTIN/CRVENE DETELINE ZA PRIMENE U MEDICINSKOM TEKSTILU

***APSTRAKT:*** *Ovde izveštavamo o stvaranju nanoemulzija (NE) pektin(P)/ekstrakt crvene deteline (RCE). U tom cilju, P/RCE NE su pripremljeni korišćenjem procesa ultrazvučne obrade (0, 1, 5 i 10 min) iu odsustvu iu prisustvu smeše surfaktanta koja se sastoji od 1/3*

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*Span® 80/Tveen® 80. Veličina kapljice i Podaci o potencijalu Zete su statistički procenjeni. Najznačajniji faktor za RCE NE je utvrđeno da je prisustvo smeše surfaktanata. Minimalna veličina kapljica (predviđanje: 51±1 nm) se može postići određivanjem vremena ultrazvučne obrade kao 5 min korišćenjem 1/3 Span® 80/Tveen® 80 za vodene RCE NE. Dodavanje RCE NE u rastvore pektina povećalo je veličinu kapljica u velikoj meri (372-728 nm). Pripremljeni P/RCE NE mogu se koristiti za oblaganje bilo koje vrste vlaknaste strukture za proizvodnju medicinskog tekstila za hormonsku regulaciju.*

***Ključne reči:*** *ekstrakt crvene deteline, pektin, surfaktant, ultrazvučna obrada, nano emulzija.*

## 1. INTRODUCTION

Emulsion is defined as the dispersion of two immiscible liquids. Here, spherical droplets form the dispersed phase, while the liquid surrounding it forms the continuous phase [15,16]. Water and oil are the commonly used liquids to form emulsions. The oil droplets dispersed in an aqueous phase are called oil-in-water (O/W) emulsions. A third component called emulsifier/surfactant is added to emulsions for both decreasing the droplet size and achieving a long-term stability. Emulsifiers have hydrophilic and hydrophobic parts, which position in the aqueous and oil phase, respectively [1,2], thereby reducing the interfacial tension and hindering/delaying the aggregation of droplets [3].
Based on their droplet size and stability, there are 3 types of emulsions, i.e., coarse emulsions, micro emulsions, and nano emulsions [4] (Table 1). Nowadays, NEs have attracted great attention due to the following advantages: They require less amount of emulsifier for their preparation. They are not affected by physical and chemical variations including temperature and pH [5]. They can encapsulate active ingredients such as herbal extracts/essential oils, endowing them with increased solubility and controlled release properties [6,7].
Herbal extracts/essential oils play a significant and increasingly important role in the food- and health-related fields [8]. However, they require emulsifiers to improve their water insoluble, unstable, and volatile natures. Pectin can act as an emulsifier in nanoemulsions (NEs) due to containing hydrophobic methoxyl and hydrophilic carboxyl groups in its structure [9,10]. Therefore, it can be very interesting to prepare pectin nanoemulsions together with herbal extracts for women’s health. Many herbs traditionally used for women's health conditions have been found to contain phyto-estrogens. During the past few decades, red clover (*Trifolium pratense L.*), being a leguminous plant with phyto-estrogenic components, has gained considerable attention.
Red clover contains some important isoflavones such as genistein, daidzein, formononetin, and Biochanin A, which have beneficial effects on prevention of osteoporosis, improved cardiovascular health and lowered risk of diabetes, obesity, neurological disorders and a wide variety of cancers including breast and endometrial cancer [11-16]. It would be interesting and beneficial to generate pectin nanoemulsions together with red clover extract

for medical textile applications. This is the first study, in which pectin/red clover extract nanoemulsions were generated and characterized.

**Table 1**: Emulsion types: physicochemical properties [17].

| | Emulsion/coarse emulsion | Micro emulsion | Nano emulsion |
|---|---|---|---|
| **Size** | 1-100 µm | 10-100 nm | <200 nm |
| **Optical property** | Turbid | Clear | Clear/slightly opaque |
| **Preparation method** | High and low energy | Low energy | High and low energy |
| **Effect of T and pH** | Stable | Affected | Stable |

## 2. EXPERIMENTAL PART

### 2.1. Materials

Pectin, dimethyl sulfoxide (DMSO) (ACS Reagent, > 99.90), Tween® 80, Span® 80, ethyl oleate, and all other chemicals and solvents were purchased from Sigma Aldrich Pty. Ltd, Australia. Red clover extract (RCE) was kindly provided by Linnea SA, Switzerland. Ultrapure (UP) water (conductivity: 0.055 µS/cm) was used in all experiments.

### 2.2. Preparation of RCE Containing Aqueous and Pectin Nanoemulsions

A total amount of 10 ml coarse emulsions containing RCE was ultrasonicated at 20 kHz, 50% power (400 Watts), 70% pulse for 0, 1, 5, 10, and 20 min. For this purpose, a stepped micro processing tip model of OR-T-156 (diameter: 3.8 mm, length: 25.6 cm) was used. Experiments were designed considering the related literature [18,19]. 50 mg RCE was used in a total volume of 10 ml emulsion. For this purpose, RCE was first dissolved in DMSO and then mixed with ethyl oleate. Afterwards, oily phase containing RCE was added to aqueous phase. In the presence of the surfactant mixture, it was mixed with the aqueous phase previously. Experimental design and related notations were given in Table 2. Stock aqueous RCE NEs were generated and stored in the fridge in aluminium foil wrapped tubes to due RCE's light sensitivity.

**Table 2**: The notation used for stock aqueous RCE NEs.

| **Explanation** | **Notation for Ultrasonication** | | | |
|---|---|---|---|---|
| | **0 min** | **1 min** | **5 min** | **10 min** |
| - surfactant mixture | SR.0 | SR.1 | SR.5 | SR.10 |
| + surfactant mixture | SRT.0 | SRT.1 | SRT.5 | SRT.10 |

To generate pectin RCE NEs, pectin solution was prepared first. Pectin (6 wt.%) was added to pre-heated ultrapure water and stirred overnight at 75°C, 1000 rpm. Pectin solutions were just vortexed after adding stock RCE NEs prepared according to the optimised processing conditions. Notation used for aqueous and pectin RCE NEs is shown in Table 3.

**Table 3**: The notation used for aqueous and pectin RCE NEs.

| Explanation | NE Type | Notation |
|---|---|---|
| - surfactant mixture | Aqueous | R.0 |
| | | R.5 |
| + surfactant mixture | | RT.0 |
| | | RT.5 |
| - surfactant mixture | Pectin | PR.0 |
| | | PR.5 |
| + surfactant mixture | | PRT.0 |
| | | PRT.5 |

**2.3. pH and Electrical Conductivity of Nanoemulsions**

The pH and electrical conductivity of solutions and nanoemulsions were measured using a pH meter (Mettler Toledo SevenEasy pH-Meter) and conductivity meter (TPS, WP-Plus), respectively.

**2.4. Dynamic Light Scattering Analysis of Nanoemulsions**

Dynamic light scattering (DLS) measurements were performed with the Zetasizer Nano ZS (Malvern Panalytical Ltd, UK).

**2.5. Statistical Analysis of DLS Results of Nanoemulsions**

The results derived from the Zetasizer Nano ZS measurements were statistically evaluated in Design Expert®6.06 software by using general factorial design. To judge the statistical importance of the variations, ANOVA was applied. Statistical significance was calculated with statistical significance level set at $p \leq 0.05$.

**3. CONCLUSION**

**3.1. pH and Electrical Conductivity of Stock Aqueous RCE NEs**

Ultrapure (UP) water has a pH value of 5.53±0.02. Stock aqueous red clover extract nanoemulsions with and without surfactant mixture showed slightly increased pH values (between 5.71±0.02 and 5.82±0.03). All stock aqueous red clover extract nanoemulsion samples had low electrical conductivity values (13.13±0.21 and 21.00±0.23 µS/cm).

**3.2. Statistical Analysis for Zeta Potential of Stock Aqueous RCE NEs**

Droplet size and Zeta potential values were statistically analysed for determining the optimum processing conditions. After entering the Zeta potential data to the Design Expert®6.06 software, LINEAR model was selected (**Table 4** and **Table 5**).

**Table 4**. Sequential model sum of squares for Zeta potential of stock aqueous red clover extract nanoemulsions.

| Source | Sum of Squares | DF | Mean Square | F Value | Prob > F | |
|---|---|---|---|---|---|---|
| Mean | 11056,52 | 1 | 11056,52 | | | |
| Linear | 340,6093 | 3 | 113,5364 | 5,022844 | 0.0175 | **Suggested** |
| 2FI | 71,70234 | 3 | 23,90078 | 1,077983 | 0.4064 | |
| Quadratic | 37,24889 | 1 | 37,24889 | 1,836086 | 0.2124 | Aliased |
| Cubic | 126,3406 | 4 | 31,58516 | 3,513726 | 0.1255 | Aliased |
| Residual | 35,95631 | 4 | 8,989079 | | | |
| Total | 11668,38 | 16 | 729,2738 | | | |

**Table 5**. Model summary statistics for Zeta potential of stock aqueous red clover extract nanoemulsions.

| Source | Std. Dev. | R-Squared | Adjusted R-Squared | Predicted R-Squared | PRESS | |
|---|---|---|---|---|---|---|
| Linear | 4,754368 | 0,556681 | 0,445851 | 0,184144 | 499,1875 | **Suggested** |
| 2FI | 4,70869 | 0,673869 | 0,456448 | -0,09975 | 672,8899 | |
| Quadratic | 4,504122 | 0,734747 | 0,502651 | -0,20668 | 738,3177 | Aliased |
| Cubic | 2,998179 | 0,941234 | 0,779628 | 0,36301 | 389,7474 | Aliased |

To judge the statistical importance of the variations, Analysis of Variance (ANOVA) was applied (**Table 6**). The model developed explains 55,66% of Zeta potential with these data.

**Table 6**. ANOVA table for Zeta potential of aqueous red clover extract nanoemulsions.

| Source | Sum of Squares | Contribution-% | DF | Mean Square | F Value | Prob > F | |
|---|---|---|---|---|---|---|---|
| Model | 340,6093 | 55,66808 | 3 | 113,5364 | 5,022844 | 0.0175 | **significant** |
| A | 165,1225 | 26,98708 | 1 | 165,1225 | 7,305007 | 0.0192 | **significant** |
| B | 90,84681 | 14,84771 | 1 | 90,84681 | 4,019056 | 0.0681 | |
| C | 84,64 | 13,83329 | 1 | 84,64 | 3,744467 | 0.0769 | |
| Residual | 271,2482 | 44,33192 | 12 | 22,60402 | | | |
| Cor Total | 611,8575 | 100 | 15 | | | | |

The regression contour plots obtained from regression equations are demonstrated in **Figure 1**. Here x-axis represents days and y-axis shows ultrasonication time.

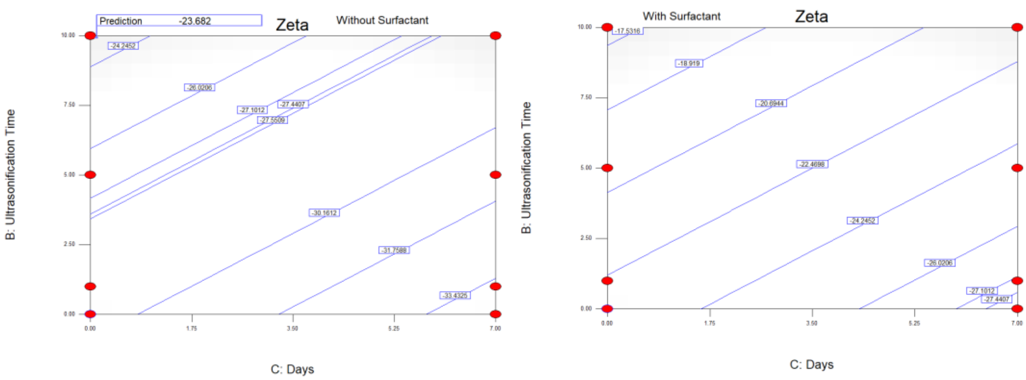


**Figure 1.** Regression contour plots for Zeta potential of stock aqueous red clover extract nanoemulsions without (left) and with (right) surfactant mixture.

### 3.3. pH and Electrical Conductivity of Aqueous and Pectin RCE NEs

Aqueous RCE NEs delivered nearly the same pH and electrical conductivity values as the stock aqueous RCE NEs. Usage of pectin solutions decreased the pH values considerably (changing between 3.70 and 3.90), which could be attributed to acidic nature of pectin. Electrical conductivity (EC) values of the pectin RCE NEs were also very high (between 2480±31 - 2589±14 mScm$^{-1}$).

### 3.4. Droplet Size Distribution Aqueous and Pectin RCE NEs

Independent of ultrasonication, red clover extract nanoemulsions (RCE NEs) without containing the surfactant mixture presented the same droplet size of around 250 nm. Addition of surfactant mixture further decreased the droplet sizes to 166 nm in the case of only vortexing. Application of an ultrasonication time of 5 min provided very small sizes of 56 nm.

Addition of the stock RCE NE to pectin solutions increased the droplet sizes to a large extent. Especially the addition of ultrasonicated samples led to bigger droplet sizes in pectin red clover extract nanoemulsions.

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