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Optimized Preparation of Eggshells Calcium Citrate（ESCC）by PEF Technology and its Accumulation in Mice Bone

Yiding Yu [1], Jingbo Liu [1] and Songyi Lin [1 +]

[1] Laboratory of Nutrition and Functional Food, Jilin University, Changchun, 130062, P.R. China

Abstract. Under optimized PEF treatment for production of Eggshells calcium citrate（ESCC） by one-factor-at-a-time test(OFAT) and ternary quadratic regression orthogonal combination design (TQROCD), the highest dissoluble calcium citrate content (7.119 mg/mL) was obtained with the 2.0 % citric acid, the electric field intensity of 15 kV/cm, and pulse duration of 20 μs. In vivo, ESCC chewable tablets prepared by the best conditions of PEF at doses of 133.0 $mg•kg^{-1}•d^{-1}$ significantly improve not only the femurs length and diameter but also organic matter of femurs and weight of the mice calcium content of bone ($P < 0.05$).

Keywords: Eggshells calcium citrate（ESCC）; Pulse electric fields (PEF); Ternary quadratic regression orthogonal combination design (TQROCD); Calcium accumulation；

1. Introduction

Calcium deficiency in the elderly and in children is a serious risk especially to those populations. Calcium citric is calcium salt of citric acid as a new resource of calcium. It used for the prevention and treatment of calcium deficiency in dietary supplements. Calcium citric promotes bone and teeth health. Calcium is more easily absorbed by human body due to its water solubility and high calcium content. Calcium citrate is water soluble, generally regarded as safe, tasteless and has high absorptive properties by the human body [1]. Pulsed electric field (PEF) is a non-thermal food-processing technology which uses short bursts of electricity (microseconds to milliseconds), providing fresh-like and safe foods and reducing quality degradative reactions that can be triggered after heat processing [2]. Until now, PEF assisted-extraction of calcium from eggshells was studied in our previous report firstly, in which mainly focused on An absorption assessment of eggshells calcium malate (ESCM) by the best conditions of PEF were performed in male mice with apparent calcium absorption rate (ACAR), serum alkalinity phosphatase (ALP), tartrate-resistant acid phosphatase (TRAP), serum calcium and serum phosphorus, length of femurs and skeletal calcium content [3]. Because eggshells calcium citrate（ESCC）is an other new calcium source to help treat and prevent calcium deficiency, optimized preparation of ESCC by PEF technology have been researched deeply. This work is focus on as following:（1）Optimized conditions were obtained by one-factor-at-a-time test (OFAT) and ternary quadratic regression orthogonal combination design (TQROCD) respectively. (2) ESCC chewable tablets are prepared by the best conditions of PEF technology. (3)Accumulations of ESCC in mice bone were evaluated at doses of 133.0 $mg•kg^{-1}•d^{-1}$.

2. Materials and Methods

2.1. Materials and instruments

Egg shells were provided from Laboratory of Nutrition and Functional Food of College of Light Industry and Economics & Management of Jilin University, and were dried, and sieved by 180-mesh. Calcium

+ Corresponding author. Tel.: +86 13304325228; fax: +86 431 87835760.
E-mail address: linsongyi730@163.com.

gluconate oral solution （CGOS）was purchased from Sanjiu Huangshi pharmaceutical factory (Huangshi, Hubei province, China). All chemicals and reagents used were of analytical grade.

A CR20B2 high speed freezing centrifuge (Hitachi Ltd, Shanghai, P. R. China), ZD-2-automatic potentiometric titrimeter (Hongyi Instrument Inc. Shanghai, P. R. China), and JY92-2D ultrasound cell smasher (Xinzhi Biotech Inc, Ningbo, P. R. China) were used. Self-designed PEF system was described in our previous report, which consisted of a high-voltage repetitive pulse generator, a coaxial liquid materials treatment chamber, a fiber-optic temperature sensor, and a data acquisition system [3]-[5].

2.2. OFAT test and TQROCD

One gram of the eggshell powder was weighed and mixed with 50 mL of acid solution of various concentrations, according to the experimental design. Then the mixture of the eggshell powder was pumped into the PEF system at a flow velocity (v) of 25 mL/min. The high voltage pulse generator was turned on, and then the pulse frequency and charge voltage were adjusted to the desired levels. After being processed for 2 min, the high voltage pulse generator was turned off, and the mixture was centrifuged to remove the solid matter [4],[5]. OFAT of PEF Treatment focuses on effects of different acids, different electric field intensities, and different pulse duration on concentration of ESCC respectively. TQROCD with three independent variables (X_1 for electric field intensity, X_2 for pulse duration, and X_3 for concentration of acid at three levels) was carried out [3]. The symbols and levels are shown in Table 1, which was based on the results of preliminary experiments. As seen from Table 2, the complete design consisted of 17 experimental points, which were obtained in a random order test. Each variable was coded at five levels: -1.35, -1, 0, +1 and +1.35. The optimal parameters obtained from previous one-factor-at-a-time were set as 0 level of each factor. Triplicates at the center (0, 0 and 0) of the design were conducted to allow the estimation of the pure error sum of squares [3].

Table1. Independent variables and their levels

x_j	Z_1	Z_2	Z_3
Factor level	Electric-field intensity (kV/cm)	Pulse duration (μs)	Concentration of citric acid (%)
1.35	20.0	20.0	3.0
1	18.7	17.8	2.7
0	15.0	12.0	2.0
-1	11.3	6.2	1.3
-1.35	10.0	4.0	1.0

Table 2. Experiment scheme and the experimental results

Factor Number	X_1	X_2	X_3	ESCC (mg/mL)	Factor Number	X_1	X_2	X_3	ESCC (mg/mL)
1	1	1	1	6.016	10	-1.35	0	0	4.897
2	1	1	-1	5.784	11	0	1.35	0	7.119
3	1	-1	1	6.888	12	0	-1.35	0	5.597
4	1	-1	-1	4.952	13	0	0	1.35	6.443
5	-1	1	1	6.919	14	0	0	-1.35	6.325
6	-1	1	-1	5.028	15	0	0	0	6.160
7	-1	-1	1	6.072	16	0	0	0	6.157
8	-1	-1	-1	5.120	17	0	0	0	6.179
9	1.35	0	0	5.294					

2.3. Determination of ESCC concentration

Calcium content was determined using the EDTA titration method [6]. The content of dissoluble calcium can be calculated by following formula:

$$Ca^{2+}\ (mg/L) = \frac{X \times (V - V_0) \times 40}{V_1} \quad (1)$$

Where X is the concentration of EDTA solution (mol / L); V is the volume of EDTA consumed by samples (mL), V_0 is the volume of EDTA consumed by blank (mL), V_1 is the volume of the sample (mL).

2.4. ESCC chewable tablets preparation

ESCC tablets were prepared according to the method described by our previous report by the Nutrition and Functional Food Lab and College of Biological and Agricultural Engineering (Jilin University, Jilin Province, R. P. China). And there were three important steps as following [3]. First, egg shells were crushed and dried, then sieved by 180-mesh. Water was added at the ratio of 10:1. The mixture was stirred 10 min and placed 20 min, then dried at 120 ℃ up until constant weight. Secondly, ESCC was prepared by PEF processing optimum conditions (15 kV/cm for the electric field strength, 10 for the total pulse number, and 2.0 % for the concentration of citric acid). Thirdly, ESCC chewable tablets were made. The ESCC solution was condensed, and then dried at 90 ℃ until constant weight was achieved up. ESCC powder was white, and contained Ca 22.27 %. ESCC powder (14 %), starch (food grade, 50.4 %), dextrin (food grade, 25.2 %) and xylitol (food grade, 8 %) were sieved by 60-mesh, and mixed uniformly. Soft material was made after adding adhesive. Soft material was made after adding the adhesivewhose composition was malic acid (food grade, 0.24 %), citric acid (food grade, 0.16 %), ascorbic acid (food grade, 1 %), milk essence (food grade, 0.5 %). Soft material was palletized with 40-mesh sieve, then dried at 50 ℃ for 2 hours and stirred once every 30 min. Then the material was sieved by 20-mesh and mixed magnesium stearate (food grade) 0.8 %. ESCC chewable tablets were made under the pressure of 3000 N. The weight was about 0.425 g per piece.

2.5. Assessment of accumulation in mice bones

Fifty male Kunming mice after physical and behavioral examination were obtained from Experimental Animal Center, Jilin University. The qualified number of mice is SCXK-(Jin) 2007-0003. The male mice weights were 10 ± 2 g at the beginning of the experiment. Animal feeds were purchased from yisheng experimental animal feed factory (Changchun, Jilin province, China). The qualified number of feed is SCXK-(Jin) 2010-0001. Following acclimatization, male mice were randomly allocated to four groups based on body weight. Male mice of negative control group (NCG, n = 10) were administrated with redistilled water (calcium content of gavages each mouse 0 $mg•kg^{-1}•d^{-1}$). Male mice of positive control group (PCG, n=10) were fed with CGOS (calcium content of gavages each mouse 133.0 $mg•kg^{-1}•d^{-1}$). Male mice of ESCC group (ESCC, n = 10) were raised with ESCC chewable tablets at dose of Calcium content of gavages 133.0 $mg•kg^{-1}•d^{-1}$). According to the method described by Toshinao G. et al. [7].The left femurs from each mouse that the muscles and connective tissues were carefully removed cleanly were determined. Bone index of the left femurs were determining their length with vernier caliper (Shanghai, China). Bone calcium of the left femurs were determining with 3 mol/L hydrochloric acid for 36 hours. The content of calcium of bone was determined with EDTA titrimetric method. The measurement method was as same as dissoluble calcium content. The amount of calcium was calculated within bone unit quality included content of calcium.

2.6. Statistical analysis

All analyses were performed using SPSS 11.5 (SPSS Inc. Chicago, IL.). All experiments were in triplicates and the means of three data sets were presented. Measurements were considered statistically different using a level of significance equal to 0.05. The significantly different was determined with $P < 0.05$. The data are expressed as mean ± relative standard deviation (R.S.D.).

3. Results and discussion

3.1. Results of OFAT

- **Effects of concentration of citric acid on calcium dissolution were shown in Fig. 1(A).**

The dissoluble ESCC contents in the control sample were increased due to the increase of the concentration of citric acid until the concentration of 3.0 %, and then gradually decreased. The dissoluble calcium contents of PEF treated sample with the same citric acid showed the ESCC concentration was the highest as 6.434 ± 0.258 mg/mL while the citric acid concentration was 3.0 %, which had significantly improved ($P < 0.05$) compared with the ESCC concentration when citric acid concentration was 2.5 %. Compared with control, all PEF treated samples significantly improved the ESCC concentration ($P < 0.05$). According to related researches, PEF could damage the fibrous protein to facilitate calcium dissolution. Similar tendency of dissoluble calcium content by PEF were reported by Yin et al [4], [5].

- **Effects of electric field intensities on calcium dissolution were shown in Fig. 1(B).**

Based on the curve of the electric field intensity versus the ESCC concentration significantly increased from 4.571±0.036 mg/mL to 5.623 ±0.032 mg/mL when electric field intensity increased from 0 to 15 kV/cm *(P < 0.05)*. The ESCC concentration with the malic acid concentration of 3.0 %, the highest content of 5.623 ± 0.032 mg/mL was found when using the electric field intensity of 15 kV/cm. Compared with non-treated sample (E = 0 kV/cm), PEF treated samples had significantly higher ESCC concentration *(P < 0.05)*. It is obvious that the PEF intensity is useful and critical in improving the calcium dissolving out from eggshell. Similar tendency changes of ESCC concentration on effects of different electric field intensities were reported by many scholars. Zeng et al. found that fatty acid content of peanut was influenced inconspicuously by the electric field intensities [8].

- **Effects of pulse duration on ESCC concentration were shown in Fig. 1(C).**

With the PEF electric pulse duration increasing from 0 μs to 12 μs, the content of dissoluble ESCC increased from 4.652 ±0.039 mg/mL to 7.015 ± 0.02 mg/mL（*P < 0.05*）. However, the contents of dissoluble ESCC were slightly decreased when the electric pulse duration changed from 16 μs to 20 μs. The To sum up the comprehensive consideration, pulse duration at 16 μs could be chosen as the best pulse parameter for PEF treatment with adding malic acids to facilitate calcium dissolution. Compared with non-treated sample (Pulse duration = 0 μs), PEF treated samples had significantly higher ESCC concentration *(P < 0.05)*. Other published reports can be found on the effect of pulse duration. The DNA extraction from bovine spleen significantly increased when pulse duration increased 0 μs to 16 μs, and then decreased with the increase of pulse duration [9]. Yin et al. also found that the content of dissoluble calcium from bone increased with the increase of pulse duration [4], [5].

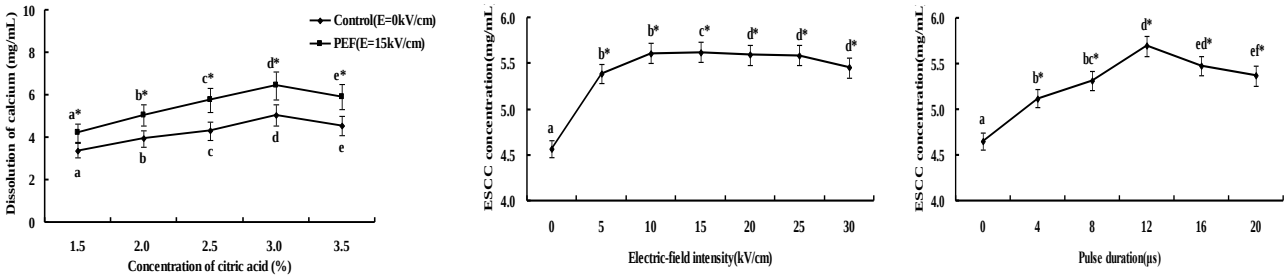

Fig. 1(A) Fig. 1(B) Fig. 1(C)

Fig. 1: Effects of concentration of acid, electric field intensity and pulse duration on ESCC concentration. Each value is expressed as means ± R.S.D. Different lowercase letters donate that variance of two samples was significant (p <0.05). * donates that variance of PEF treated sample and control was significant (p <0.05).

3.2. Results of TQROCD

A total of 17 experimental runs for three kind of calcium organic acid were performed for optimizing the three individual variables. The results of TQROCD were shown in Table 2 and Table 3. The regression equation model for the concentration of ESCC was: $Y=4.513 + 0.216 X_1^2 - 0.098 X_2^2 + 0.453 X_2 + 0.215 X_3 + 0.316 X_1X_2$. Here, X_1, X_2 and X_3 were the variables for the concentration of citric acid (m/m), the electric-field intensity (kV/cm) and the total pulse number, respectively. The F-value of the model was significant ($P < 0.05$), while the lack of fit value of the mathematical model was 1.477, which meant that the model does not lack fit. Using the analysis of variance, the significance level of the regression equation was 0.01. Therefore, it was the optimized regression for ESCC. Therefore the PEF processing optimum conditions were 15 kV/cm for the electric field strength, pulse duration of 20 μs, and 2.0 % for the concentration of citric acid. Under the optimized conditions, the highest concentration of dissoluble calcium was 7.12 mg/mL.

3.3. Effects on accumulation in mice bone

Effects on accumulation in mice femur were shown in Table 4. The lengths of left femurs of NCG had no significant differences ($P > 0.05$). The femurs length of the mice fed with ESCC were significantly greater than those from NCG ($P < 0.05$). There were not significant differences existed among the ESCC and PCG in the lengths of the femurs ($P > 0.05$). Left femurs diameters of ESCC had significant differences with NCG ($P < 0.05$). ESCC treatment significantly increased weight of left femurs compared to NCG ($P < 0.05$).

Left femur weights of ESCC had no significant differences ($P > 0.05$) with PCG. The calcium content of bone had no significant differences between NCG ($P > 0.05$). Calcium content bone of ESCC was higher than that of NCG and PCG ($P < 0.05$). The organic matters of femur were no significant differences between ESCC and NCG ($P > 0.05$). While ESCC chewable tablets improve not only the femurs length and diameter but also organic matter of femurs and weight of the mice calcium content of bone significantly ($P < 0.05$).

Table 3. Results of variance of regression model

Variance Source	Square sum SS	Degree of freedom df	Mean square MS	*F Value*	*P*
Model	5.243	4	1.311	39.421	0.01
Residual	0.399	12	0.033		
Lack of fit	0.342	10	0.034	1.200	0.25
Pure error	0.057	2	0.029		
Cor. Total	5.642	16	0.353		

PS： $F_{0.01}(5,11)=5.32$, $F_{0.25}(9,2)=3.37$, $F_{0.01}(4,12)=5.41$, $F_{0.25}(10,2)=3.38$, $F_{0.01}(3,13)=5.74$, $F_{0.25}(11,2)=3.38$.

Table 4. Effects on accumulation in mice bone (n = 10, 70 days)

Groups	Left femur Length (cm)	Left femur Diameter (cm)	Weight (g)	Calcium content of bone (mg/g)	Organic matter of femur (%)
NCG	1.493±0.113	0.226±0.143	0.091±0.125	147.61±0.282	16.24±0.121
PCG	1.639±0.027*	0.252±0.095	0.098±0.102	179.19±0.177	18.36±0.132
ESCC	1.635±0.092*	0.242±0.073*	0.102±0.097*	181.701±0.158*#	18.19±0.137*

Each value is expressed as means ± R.S.D. ***** showed the significant difference compared with NCG (P <0.05). **#** showed the significant difference with PCG (P <0.05).

4. Conclusions

By PEF technology, the highest dissoluble ESCC content calculated was 7.12 mg/mL with the best processing conditions of ESCC as 2.0 % citric acid, 15 kV/cm for electric field intensity, and pulse duration of 20 μs. By male mice tests, compare with NCG group and PCG group, the femurs length ,diameter ,organic matter of femurs, and weight of the mice bone calcium content of ESCC group fed ESCC chewable tablets at doses of 133.0 $mg•kg^{-1}•d^{-1}$ were improved significantly ($P < 0.05$).

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