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Optimization of Lycopene Extraction from Tomato Cell Suspension Culture by Response Surface Methodology

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Radioisotope-labeled lycopene is an important tool for biomedical research but currently is not commercially available. A tomato cell suspension culture system for the production of radioisotope-labeled lycopene was previously developed in our laboratory. In the current study, the goal was to optimize the lycopene extraction efficiency from tomato cell cultures for preparatory high-performance liquid chromatography (HPLC) separation. We employed response surface methodology (RSM), which combines fractional factorial design and a second-degree polynomial model. Tomato cells were homogenized with ethanol, saponified by KOH, and extracted with hexane, and the lycopene content was analyzed by HPLC-PDA. We varied five factors at five levels: ethanol volume (1.33−4 mL/g); homogenization period (0−40 s/g); saturated KOH solution volume (0−0.67 mL/g); hexane volume (1.67−3 mL/g); and vortex period (5−25 s/g). Ridge analysis by SAS suggested that the optimal extraction procedure to extract 1 g of tomato cells was at 1.56 mL of ethanol, 28 s homogenization, 0.29 mL of KOH, 2.49 mL of hexane, and 17.5 s vortex. These optimal conditions predicted by RSM were confirmed to enhance lycopene yield from standardized tomato cell cultures by more than 3-fold.

KEYWORDS: Tomato; cell culture; lycopene; extraction; RSM

INTRODUCTION

Increased consumption of tomato and tomato products has been significantly associated with a reduced risk of prostate cancer in several epidemiological studies (*1*). Carotenoids are yellow, orange, and red pigments present in fruits and vegetables, which possess a wide range of proposed biological functions, including antioxidant, anticarcinogen, and immunoprotective properties. Lycopene, the most abundant tomato carotenoid, has been the primary focus of both in vitro and in vivo studies examining the relationship between increased intake of tomatoes and reduced risk of prostate cancer. Numerous epidemiologic studies have shown that higher serum lycopene concentration is inversely related to prostate cancer risk (*2*−*4*).

To study their modes of action, radiolabeled carotenoids are indispensable tools and are often used to trace the absorption, distribution, metabolism, and elimination of the dietary compound of interest (*5*). There exists a keen interest in the influence of tomato carotenoids on the risk of prostate cancer (*1, 6, 7*); however, radiolabeled carotenoids such as lycopene and its precursors are not commercially available. Therefore, a tomato cell suspension culture system was developed in our laboratory to biosynthesize and radiolabel tomato carotenoids for in vitro prostate cancer cell studies (*8*). In this system, ^{14}C-labeled sucrose was used as a carbon source, and the herbicide, norflurazon, was added to the cell suspension culture to induce biosynthesis and maximize accumulation of carotenoids. These radiolabeled carotenoids were successfully produced, but the recovery of radiolabeled carotenoids did not meet our expectation. Multiple steps such as cell destruction, lipid removal, and liquid−liquid partition could influence the yield during extraction process, and the interaction between these factors could be very complex. Therefore, a sophisticated statistical method is necessary for optimizing this extraction method.

Response surface methodology (RSM) is a useful statistical technique, which combines fractional factorial design and a second-degree polynomial model to investigate complex processes, and it has been widely used in different fields. The original concept was developed by Box and Wilson (*9*), and the basic theoretical, fundamental, and biological applications were reviewed by Mead and Pike (*10*). Our goal was to optimize the lycopene extraction procedure by applying the response surface methodology and to further maximize the radiolabeled carotenoid isolation from tomato cells for use in prostate cancer research.

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MATERIALS AND METHODS

Materials. Lycopene standard was a gift from DSM Nutritional Products (Kaiseraugst, Switzerland). High-performance liquid chromatography (HPLC) solvents were purchased from Fisher Scientific (Fair Lawn, NJ). All reagents were of analytical grade.

Tomato Cell Suspension Culture. Tomato callus was induced from sepal explants of greenhouse-grown tomato plants, *Lycopersicon esculentum* cv. VFNT Cherry, on agar-solidified medium as previously described (*8*). Briefly, the medium used for callus induction contained Murashige and Skoog basal salts, Nitsch's vitamins, myo-inositol (100 mg/L), and 3% sucrose, supplemented with plant growth regulators 2,4-dichlorophenoxyacetic acid (2 mg/L) and 6-benzylaminopurine (0.1 mg/L) and solidified with agar. Once friable callus was obtained, approximately 2.0 g of callus was transferred to 40 mL of liquid medium identical to callus induction media.

Solution cultures were transferred to a carotenoid production media in 250 mL Erlenmeyer flasks containing the plant growth regulators indole-3-acetic acid (5 mg/L) and *all-trans*-zeatin (2 mg/L) and placed on a rotary shaker at 160 rpm. Cultures were continuously maintained on this media by regularly subculturing 4 mL packed cells and 8 mL of spent media to fresh media every 2 weeks. For carotenogenesis induction, 2-(4-chlorophenylthio)triethylamine (CPTA) (0.075 g/L) was added on day one of the growth cycle. After a 2 week growth cycle, cells were harvested and separated from the growth media using Whatman no. 4 filter paper and gentle vacuum until no liquid was expressed for 30 s. Collected cells were mixed before sampling to minimize variation, stored under argon, and frozen at −80 °C until extraction. Two batches of tomato cells were used in this study: the first batch was used for model construction, and the second batch was only used in the verification experiments.

Lycopene Extraction. The extraction method was modified from our original method, which was developed and used for tomato cell extraction (*8*). Lycopene was extracted from tomato cells by placement of 3 g of cells and 4−12 mL of ethanol with 0.1% butylated hydroxytoluene (BHT), into a 35 mL centrifuge tube. Samples were thoroughly mixed on a vortex at level 8 (Vortex, model G560; Scientific Industries, Bohemia, NY), homogenized at level 7 for 0−120 s (Homogenizer, Kinematica PCU1; Brinkmann, Westbury, NY), and then saponified by 0−2 mL of saturated KOH solution immersed in a 60 °C water bath. Subsequently, 2 mL of deionized water and 5−9 mL of hexane were added, and samples were mixed at vortex level 8 for 15−75 s and then centrifuged for 10 min at 4 °C (Centrifuge, model CR3i; Jouan, Winchester, VA). The hexane phase was removed and retained. The process of hexane addition, mixing, and centrifugation was repeated three times. Extracts were pooled and dried in a Speedvac evaporator (model 160; Savant, Farmingdale, NY), flushed with argon, and stored in a −20 °C freezer less than 24 h prior to the HPLC-PDA analysis. The whole process was performed under yellow light.

HPLC Analysis. Lycopene was analyzed by a reverse-phase HPLC-PDA system. The system consisted of a Rainin Dynamics gradient pump (model SD-200; Varian, Walnut Creek, CA), a Prostar pump (model 210; Varian), a C30 column (4.6 mm × 150 mm, 3 μm, YMC, Wilmington, NC) with a precolumn, a photodiode array detector (model 2996; Waters, Milford, MA), and Millennium^{32} software (Waters). Solvent A consisted of 83% methanol, 15% methyl-*tert*-butyl ether (MTBE), and 2% ammonium acetate aqueous solution (1.5%). Solvent B consisted of 8% methanol, 90% MTBE, and 2% ammonium acetate aqueous solution (1.5%). The gradient procedure at a flow rate of 1 mL/min was as follows: 10% B hold for 5 min, 12 min linear gradient to 65% B, 12 min linear gradient to 95% B, 5 min hold at 95% B, 2 min linear gradient to 10% B, and 2 min hold at 10% B for a final time of 38 min. The column was maintained at room temperature, and the detector was set at 472 nm. All analyses were performed in duplicate, and the quantification of lycopene was carried out with analytical standard (DSM, λ_{max} = 472 nm, $A_{1\ cm}^{1\%}$ = 3450 in hexane).

Experimental Design. A central composite design (*11*) was used to investigate the effects of five independent variables, ethanol volume (X_1), homogenization duration (X_2), KOH volume (X_3), hexane volume (X_4), and vortex duration (X_5) on the yield of lycopene (Y). The independent variables were coded at five levels (−2, −1, 0, 1, and 2), and the complete design consisted of 32 experimental points including six replications of the center points (all variables were coded as zero) (**Table 1**). The 32 sets of experiments were performed in a random order.

Table 1. Original and Coded Levels of Independent Variables

		coded				
variable	original	−2	−1	0[a]	1	2
ethanol	X_1 (mL/g)	1.333	2	2.667	3.333	4
homogenization	X_2 (s/g)	0	10	20	30	40
KOH	X_3 (mL/g)	0	0.167	0.333	0.5	0.667
hexane	X_4 (mL/g)	1.667	2	2.333	2.667	3
vortex	X_5 (s/g)	5	10	15	20	25

[a] Central point.

Statistical analysis. The experimental data were fitted to the following second-order polynomial equation by statistical analysis system (SAS Institute, Cary, NC) through the response surface regression (RSREG) procedure:

$$Y = A_0 + \sum_{i=1}^{5} A_i X_i + \sum_{i=1}^{5} A_{ii} X_i^2 + \sum_{i=1}^{4} \sum_{j=i+1}^{5} A_{ij} X_i X_j$$

where Y is the response (lycopene content, nmol/g), A_0, A_i, A_{ii}, and A_{ij} are constant coefficients, and X_i is the uncoded independent variable. The model was predicted through regression analysis and analysis of variance (ANOVA). Response surfaces were developed using fitted polynomial equations in SAS. The optimal extraction conditions for maximized lycopene yield were pre-established by ridge analysis (*11*) (RIDGE MAX procedure in SAS).

Verification of Method Improvement. The degree of method improvement was determined by comparing the lycopene yield of the optimal extraction conditions as predicted by SAS (RSM Method) and the central-point extraction conditions (central-point method), in which all variables were coded as zero (**Table 1**). We also compared these two methods with our original method (original method), which was previously developed and used for tomato cell extraction (*8*). Fisher's least-significant-difference test was applied for the comparison of these three methods.

RESULTS AND DISCUSSION

Model Fitting. Lycopene yields of 32 sets of variable combinations were obtained and analyzed (**Table 2**) by HPLC analysis and fitted into a second-order polynomial equation by an RSREG procedure. Estimated values of regression coefficients were also obtained (**Table 3**), and the regression model was predicted as follows:

$$\begin{aligned} Y = 63.85 - 17.61X_1 + 0.69X_2 + 74.52X_3 - 36.09X_4 - \\ 0.71X_5 + 5.037{X_1}^2 - 0.009{X_2}^2 - 27.409{X_3}^2 + 5.523{X_4}^2 - \\ 0.006{X_5}^2 - 0.309X_1X_2 + 0.563X_1X_3 - 0.844X_1X_4 - \\ 0.356X_1X_5 - 0.563X_2X_3 + 0.394X_2X_4 + 0.006X_2X_5 - \\ 19.125X_3X_4 - 0.375X_3X_5 + 0.863X_4X_5 \end{aligned}$$

The predicted values of lycopene yield were calculated by using the predicted regression model and compared with experimental values (**Table 2**). The value for the coefficient of determination (R^2) was 0.88, which indicates adequacy of the applied model. The statistical analysis showed that the total model, linear component, and quadratic component were all significant (**Table 4**). The analysis of variance also showed that there was a nonsignificant lack of fit, which further validates the model.

Analysis of Response Surfaces. Response surface graphs were plotted between two independent variables while remaining independent variables were kept at the zero coded level. The

Table 2. Experimental Design (Uncoded) and Response Values

	factor					response (nmol/g)	
no.	X_1	X_2	X_3	X_4	X_5	observed	predicted
1	−1 (2.000)	−1 (10)	−1 (0.167)	−1 (2.000)	1 (20)	11	11
2	1 (3.333)	−1 (10)	−1 (0.167)	−1 (2.000)	−1 (10)	11	11
3	−1 (2.000)	1 (30)	−1 (0.167)	−1 (2.000)	−1 (10)	19	20
4	1 (3.333)	1 (30)	−1 (0.167)	−1 (2.000)	1 (20)	11	11
5	−1 (2.000)	−1 (10)	1 (0.500)	−1 (2.000)	−1 (10)	11	13
6	1 (3.333)	−1 (10)	1 (0.500)	−1 (2.000)	1 (20)	9	10
7	−1 (2.000)	1 (30)	1 (0.500)	−1 (2.000)	1 (20)	19	20
8	1 (3.333)	1 (30)	1 (0.500)	−1 (2.000)	−1 (10)	11	12
9	−1(2.000)	−1 (10)	−1 (0.167)	1 (2.667)	−1 (10)	9	8
10	1 (3.333)	−1 (10)	−1 (0.167)	1 (2.667)	1 (20)	13	11
11	−1 (2.000)	1 (30)	−1 (0.167)	1 (2.667)	1 (20)	33	31
12	1 (3.333)	1 (30)	−1 (0.167)	1 (2.667)	−1 (10)	17	15
13	−1 (2.000)	−1 (10)	1 (0.500)	1 (2.667)	1 (20)	13	13
14	1 (3.333)	−1 (10)	1 (0.500)	1 (2.667)	−1 (10)	8	8
15	−1 (2.000)	1 (30)	1 (0.500)	1 (2.667)	−1 (10)	18	18
16	1 (3.333)	1 (30)	1 (0.500)	1 (2.667)	1 (20)	14	13
17	−2 (1.333)	0 (20)	0 (0.333)	0 (2.333)	0 (15)	29	28
18	2 (4.000)	0 (20)	0 (0.333)	0 (2.333)	0 (15)	16	17
19	0 (2.667)	−2 (0)	0 (0.333)	0 (2.333)	0 (15)	4	3
20	0 (2.667)	2 (40)	0 (0.333)	0 (2.333)	0 (15)	16	16
21	0 (2.667)	0 (20)	−2 (0.000)	0 (2.333)	0 (15)	9	11
22	0 (2.667)	0 (20)	2 (0.667)	0 (2.333)	0 (15)	12	9
23	0 (2.667)	0 (20)	0 (0.333)	−2 (1.667)	0 (15)	18	14
24	0 (2.667)	0 (20)	0 (0.333)	2 (3.000)	0 (15)	14	17
25	0 (2.667)	0 (20)	0 (0.333)	0 (2.333)	−2 (5)	12	11
26	0 (2.667)	0 (20)	0 (0.333)	0 (2.333)	2 (25)	14	15
27	0 (2.667)	0 (20)	0 (0.333)	0 (2.333)	0 (15)	15	13
28	0 (2.667)	0 (20)	0 (0.333)	0 (2.333)	0 (15)	10	13
29	0 (2.667)	0 (20)	0 (0.333)	0 (2.333)	0 (15)	14	13
30	0 (2.667)	0 (20)	0 (0.333)	0 (2.333)	0 (15)	8	13
31	0 (2.667)	0 (20)	0 (0.333)	0 (2.333)	0 (15)	17	13
32	0 (2.667)	0 (20)	0 (0.333)	0 (2.333)	0 (15)	15	13

Table 3. Regression Coefficients of the Predicted Quadratic Polynomial Model

parameter	estimate	standard error	*t* value	*p* value
A_0	63.85	50.22	1.27	0.230
A_1	−17.61	12.59	−1.4	0.190
A_2	0.69	0.78	0.89	0.393
A_3	74.52	46.72	1.59	0.139
A_4	−36.09	29.63	−1.22	0.249
A_5	−0.71	1.69	−0.44	0.669
A_{11}	5.037	1.384	3.64	0.004
A_{22}	−0.009	0.006	−1.44	0.178
A_{33}	−27.409	22.147	−1.24	0.242
A_{44}	5.523	5.537	1.00	0.340
A_{55}	−0.006	0.025	−0.22	0.829
A_{12}	−0.309	0.125	−2.48	0.031
A_{13}	0.563	7.497	0.08	0.942
A_{14}	−0.844	3.749	−0.23	0.826
A_{15}	−0.356	0.250	−1.43	0.182
A_{23}	−0.563	0.500	−1.13	0.284
A_{24}	0.394	0.250	1.58	0.143
A_{25}	0.006	0.017	0.38	0.715
A_{34}	−19.125	14.994	−1.28	0.228
A_{35}	−0.375	1.000	−0.38	0.715
A_{45}	0.863	0.500	1.73	0.112

Table 4. Analysis of Variance for the Second-Order Response Surface Model

source of variation	DF	sum of squares
model	20	891.8[b]
linear	5	490.2[a]
quadratic	5	214.4[b]
cross-product	10	187.1[c]
lack of Fit	6	63.3[c]
pure error	5	58.8
total error	11	122.1
R^2	0.88	

[a] Significant at 1% level. [b] Significant at 5% level. [c] Not significant.

relationship between variables is illustrated by these response surface plots.

Lycopene yield was increased with either decreased or increased ethanol volume (**Figure 1a** and **b**). At the low level of ethanol volume, predicted lycopene yield increased with lengthened homogenization period. This might be due to a higher cell concentration, which allows more cells to pass through the homogenizer per unit of time, and therefore, more cell structures could be destroyed and more lycopene would be available to be extracted. On the other hand, at a higher level of ethanol volume, the predicted lycopene yield did not vary much with the changes of homogenization period or KOH volume.

A longer homogenization period led to a higher lycopene yield (**Figure 1a** and **c**) and is most likely due to enhanced rupture of tomato cell walls. In raw tomato, lycopene is located in the chromoplasts where it appears as crystals, needlelike structures, or oily droplets, depending on the tomato variety or cultivar (*12*). As more cell structural components are destroyed, lycopene should be more accessible for hexane extraction.

In animal tissue carotenoid extraction, saponification is widely used to remove lipids and results in a better separation and a less complicated extract. Therefore, the effect of saponification was tested in this study. The predicted yield of lycopene was increased as saturated KOH solution volume increased (**Figure 1b**, **d**, and **e**), but it decreased at the high level of KOH. Although the saponification did not significantly increase the lycopene yield in the regression model, the final extract was in a dried form instead of an oily form due to the removal of lipids.

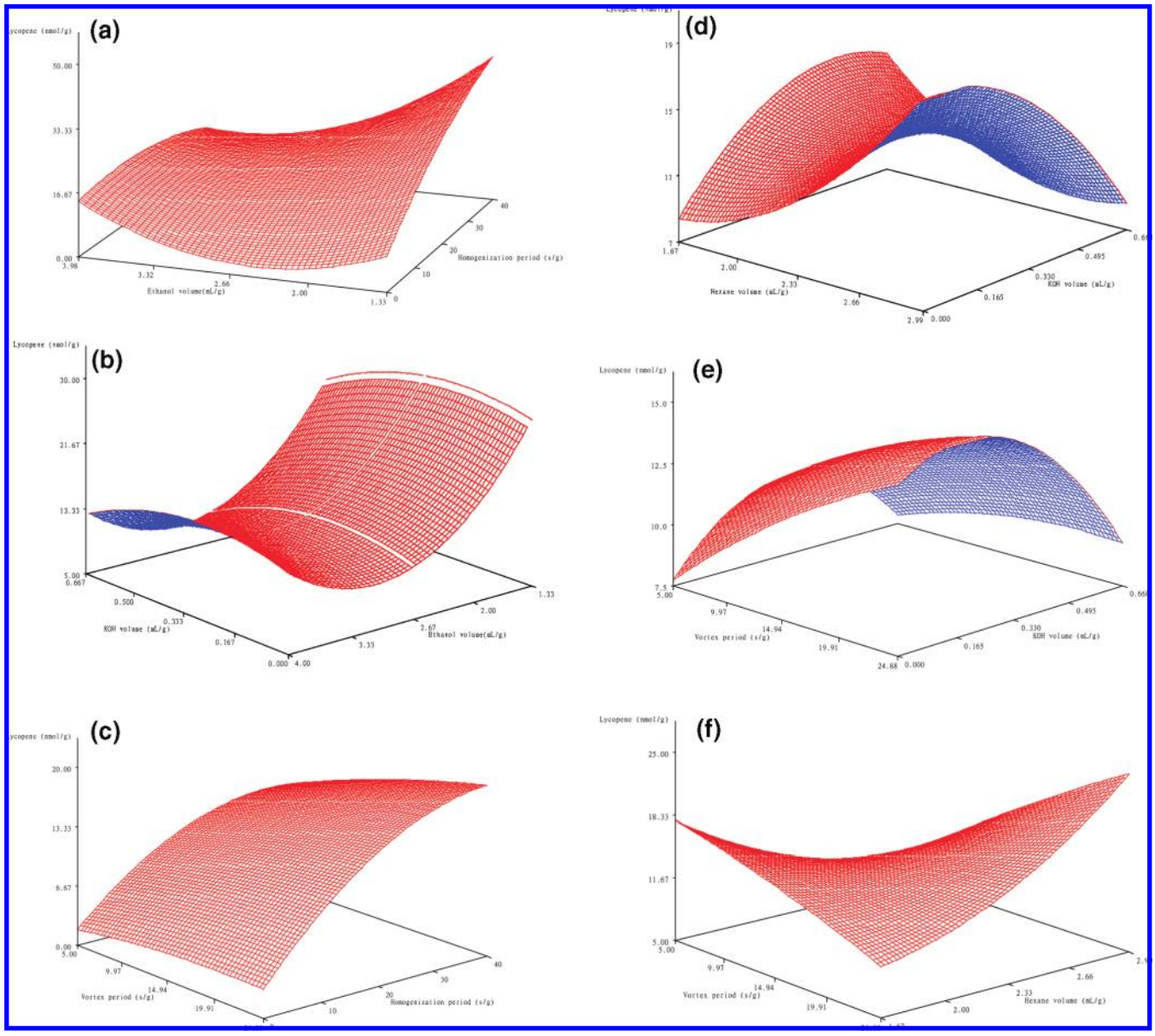

Figure 1. Response surface plots showing the effects of variables in the yield of lycopene (nmol/g tomato cells, *y*-axis): (**a**) Ethanol volume (*x*-axis) and homogenization period (*z*-axis); (**b**) KOH volume (*x*-axis) and ethanol volume (*z*-axis); (**c**) Vortex period (*x*-axis) and homogenization period (*z*-axis); (**d**) Hexane volume (*x*-axis) and KOH volume (*z*-axis); (**e**) Vortex period (*x*-axis) and KOH volume (*z*-axis); (**f**) Vortex period (*x*-axis) and hexane volume (*z*-axis).

Nonoily extracts are preferential for better HPLC separation and reduced HPLC column obstruction.

Hexane was used as the extraction solvent in this study. Although solvent mixtures that contain hexane, acetone, and ethanol have often been used in different studies and have been suggested to be of higher extraction efficiency (*13*), these mixtures also require a longer time to evaporate the solvent mixture than hexane. Lycopene yield increased as the volume of hexane and the period of vortex increased (**Figure 1f**), and similar trends were also observed in other studies (*14, 15*). It is noteworthy that Periago and coworkers (*13, 14*) also evaluated optimizing lycopene extraction from tomato and tomato products, although they did not use the response surface methodology.

Optimization and Verification. The optimum extraction condition was determined by the ridge maximum analysis. Ridge analysis generates the estimated ridge of maximum response for increasing radii from the center of original design (*11*). The ridge maximum analysis predicted that the conditions of 1.56 mL/g ethanol, 28 s/g homogenization, 0.29 mL/g KOH solution, 2.49 mL/g hexane, and 17.5 s/g vortex would lead to the maximum lycopene yield.

The verification experiment was performed in quadruplicate on a second batch of tomato cells by extracting and determining lycopene content using three extraction conditions: our original method (5 mL/g ethanol, 5 s/g homogenization, 0 mL/g KOH solution, 6 mL/g hexane, and 30 s/g vortex period) (*8*), central-point method (2.67 mL/g ethanol, 20 s/g homogenization, 0.33 mL/g KOH solution, 2.34 mL/g hexane, and 15 s/g vortex period), and the RSM method (1.56 mL/g ethanol, 28 s/g homogenization, 0.29 mL/g KOH solution, 2.49 mL/g hexane, and 18 s/g vortex period) (**Figure 2**). It was determined that the lycopene yield following the RSM method was increased 3.7-fold compared to the lycopene yield using the original method and 1.4-fold compared to the lycopene yield from the central-point method. These results confirm that RSM method conditions were significantly enhanced for lycopene extraction from tomato cell cultures. In conclusion, through the response

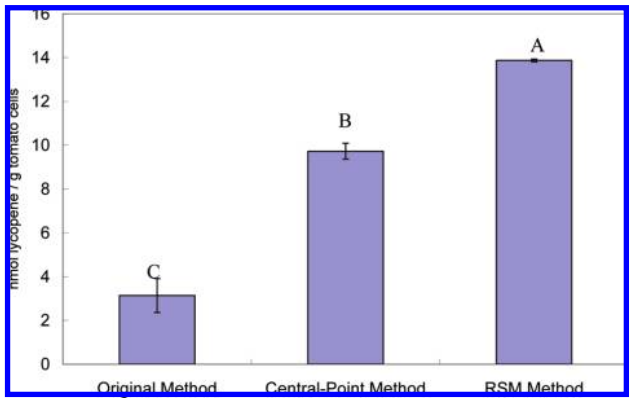

Figure 2. Effect of extraction method type on lycopene recovery from tomato cell culture. Different superscript letters on bars indicate significant differences between treatments ($n = 4$, $p < 0.005$, t test). Original method: 5 mL/g ethanol, 5 s/g homogenization, 0 mL/g KOH solution, 6 mL/g hexane, and 30 s/g vortex period. Central-point method: 2.67 mL/g ethanol, 20 s/g homogenization, 0.33 mL/g KOH solution, 2.34 mL/g hexane, and 15 s/g vortex period. RSM method: 1.56 mL/g ethanol, 28 s/g homogenization, 0.29 mL/g KOH solution, 2.49 mL/g hexane, and vortex 18 s/g vortex period.

surface methodology, the optimization of extraction procedure to maximize the lycopene yield from tomato cell suspension culture was achieved. The optimized conditions allow for over 3-fold higher yields of lycopene.

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